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PRODUCTION AND PROPERTIES OF 2,3-BUTANEDIOL

VII. FERMENTATION OF WHEAT BY *AEROBACILLUS POLYMYXA* UNDER AEROBIC AND ANAEROBIC CONDITIONS[1]

BY G. A. ADAMS[2]

Abstract

Aeration by mechanical agitation of 15% wheat mash fermented by *Aerobacillus polymyxa* inhibited the formation of 2,3-butanediol and particularly of ethanol. Aeration of similar mashes by passage of finely dispersed air or oxygen at the rate of 333 ml. per minute per litre of mash increased the rate of formation and yield of 2,3-butanediol but inhibited ethanol formation. However, the over-all time required for the completion of fermentation was not shortened from the usual 72 to 96 hr. required for unaerated mashes. There was no evidence of a shift from fermentative to oxidative dissimilation. Under aerobic conditions, the final butanediol–ethanol ratio was approximately 3 : 1. Anaerobic conditions, as produced by the passage of nitrogen or hydrogen through the mash, increased the rate of formation of both butanediol and ethanol and shortened the fermentation time to about 48 hr. Under these conditions, the butanediol–ethanol ratio was reduced to about 1.3 : 1.0. Carbon dioxide gave a butanediol–ethanol ratio resembling that of anaerobic fermentation but did not reduce fermentation time.

Introduction

Fermentation of carbohydrates by *Aerobacillus polymyxa* yields 2,3-butanediol and ethanol as the main products in a ratio of approximately 1.7 : 1.0 by weight. Since diol is the more valuable product it is desirable to obtain as high a proportion of it as possible without decreasing the total products. It is well known that vigorous aeration of *Aerobacter aerogenes* cultures stimulates production of diol. Assuming that ethanol is formed by reduction of acetaldehyde its formation by *Aerobacillus polymyxa* should be inhibited by aeration if oxygen is capable of competing with acetaldehyde as a hydrogen acceptor.

A study by Stahly and Werkman (4) on the mechanism of acetoin formation by *A. polymyxa* showed that under aerobic conditions there was a fourfold increase in acetoin, no gain in diol, and a marked decrease in ethanol as compared with anaerobic conditions. Adams and Stanier (2) have reported the results of comparative fermentations of glucose by *A. polymyxa* in atmospheres of air and nitrogen. Ethanol formation was depressed but no

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[2] *Biochemist, Industrial Utilization Investigations.*

appreciable increase in acetoin was found under aerobic conditions. Analyses performed at 10-hr. intervals showed a slightly slower fermentation in air.

The experiments reported in the present paper were undertaken to provide more information on the effects of aerobic and anaerobic conditions on the fermentation of wheat by *A. polymyxa*. In particular, means were sought for attaining a higher diol–ethanol ratio.

Wheat mashes of a concentration normally used in commercial fermentation processes instead of dilute sugar solutions were fermented in all experiments involving *A. polymyxa*. This medium permitted study of such factors as aeration, mixing, and foaming, as well as fermentation efficiency of the organism on a complex and concentrated substrate. In view of the results obtained with *Aerobacillus* fermentations, similar experiments involving air and nitrogen treatments were conducted with another diol-producing organism, *Aerobacter aerogenes*.

Materials and Methods

Fifteen per cent wheat mashes containing 1% of calcium carbonate served as fermentation media. In all experiments described in this paper two strains of *A. polymyxa*, N.R.C. C4 (2) and C3 (2), respectively, from our own collection, were used. The mashes were inoculated with a culture of the organism, brought up in two 24-hr. stages on whole-wheat–yeast-extract–calcium-carbonate media. An amount of inoculum equal to 3% of the volume of the mash was found to be sufficient. All mashes were fermented at 30° C. The methods of analysis for butanediol, ethanol, acetoin, and organic acids have been previously described (5).

Aeration by Agitation

A simple and effective method of aerating bacterial cultures during fermentation is by means of agitation on a mechanical shaker. Since this procedure has been used with good results on *Aerobacter aerogenes**, it was chosen as a means of aerating *Aerobacillus polymyxa*.

Continuous Agitation

Four hundred millilitres of 15% whole wheat mash in 2000-ml. flasks was agitated on a mechanical shaker throughout the experiment. The shaker has a total linear excursion of 2 in. at the rate of 180 cycles per minute. The mashes were inoculated with the two strains, each in duplicate, and for all experiments unshaken controls were included. Duplicate sets of flasks were removed from the shaker at 72 and 96 hr., respectively, and along with their unshaken controls were analysed for diol and ethanol.

It is apparent from the average results in Table I that aeration produced by agitation inhibited the formation of diol and ethanol by both organisms. The relatively higher diol–ethanol ratios in the agitated mashes show that the

* *Johnson, M. J., Department of Biochemistry, University of Wisconsin. Private communication.*

TABLE I

EFFECT OF AERATION BY AGITATION ON THE FERMENTATION OF 15% WHOLE WHEAT MASH BY STRAINS OF *A. polymyxa*

Treatment	Fermentation period, hr.	Butanediol, %		Ethanol, %		Total products, %		Ratio	
		N.R.C. C3 (2)	N.R.C. C4 (2)	N.R.C. C3 (2)	N.R.C. C4 (2)	N.R.C. C3 (2)	N.R.C. C4 (2)	N.R.C. C3 (2)	N.R.C. C4 (2)
None	72	2.66	2.50	1.46	1.24	4.12	3.74	1.82	2.01
Agitation	—	2.14	1.59	0.87	0.49	3.01	2.08	2.46	3.25
None	96	2.85	2.86	1.46	1.39	4.31	4.25	1.95	2.06
Agitation	—	2.80	2.30	1.26	0.67	4.06	2.97	2.22	3.43

formation of ethanol is depressed more than that of diol. While it is impossible to segregate the individual effects of mixing and aeration in this experiment, the inhibition of ethanol formation would seem to be due to aeration, since it has been shown that exposure of large surfaces of mash to air has the same effect (1). The response of the two organisms to agitation is different, C4 (2) being inhibited to a greater extent than C3 (2). The overall inhibitory effect on both organisms is less marked at 96 than at 72 hr., since the fermentations are then approaching completion.

Intermittent Agitation

Since prolonged agitation of the fermentation mashes was not beneficial, it was decided to investigate the effect of agitation followed by a period of quiescence. It is well known that in yeast fermentations an initial aeration period increases the number of organisms, and it was thought that if a similar condition could be brought about in *A. polymyxa* fermentations the increased numbers would lead to a more rapid fermentation.

Mashes were shaken for 0, 12, 24, 36, 48, 60, 72, 84, and 96 hr. At the end of each agitation period the flasks were removed from the shaker and allowed to stand for the remainder of the fermentation time. Duplicate mashes were used and analyses for diol and ethanol made at 72 and 96 hr. The fermentation was carried out with *A. polymyxa* C4 (2).

The results of 72-hr. fermentations given in Table II show that any period of agitation is detrimental in so far as total yield of diol and ethanol is concerned. Increasing the agitation time caused a progressive rise in the diol–ethanol ratio and indicated that the inhibitory effect is more pronounced on ethanol than on diol formation. It is interesting to note that diol production was most markedly repressed by a shaking period of about 48 hr., while, on the other hand, ethanol was inhibited almost uniformly by any shaking time greater than 12 hr. After fermentation for 96 hr., the inhibitory effects were less marked, especially on diol yield, which was almost as high as that of the control. The ethanol was not much greater than at 72 hr. It appears that the mechanism of ethanol formation was permanently inhibited, while the diol mechanism became partially adapted to the conditions.

TABLE II

EFFECT OF INTERMITTENT AGITATION ON THE FERMENTATION OF 15% WHOLE WHEAT MASH BY *A. polymyxa* N.R.C. C4 (2)

Treatments		Analyses at 72 hr.			
Agitation time, hr.	Standing time, hr.	Butanediol, %	Ethanol, %	Total products, %	Ratio
0	72	2.51	1.36	3.87	1.85
12	60	2.36	1.18	3.54	2.00
24	48	1.83	0.70	2.53	2.62
36	36	1.77	0.61	2.38	2.93
48	24	1.44	0.50	2.06	3.08
60	12	1.98	0.62	2.60	3.17
72	0	2.22	0.64	2.87	3.45
		Analyses at 96 hr.			
0	96	2.92	1.60	4.52	1.82
84	12	2.70	0.80	3.50	3.38
96	0	2.83	0.75	3.58	3.77
Standing time, hr.	Agitation time, hr.	Analyses at 72 hr.			
0	72	2.31	0.61	2.92	3.76
12	60	2.37	0.75	3.12	3.17
24	48	2.39	0.79	3.18	3.03
36	36	2.68	1.15	3.83	2.33
48	24	2.94	1.43	4.37	2.06
60	12	2.94	1.53	4.47	1.93
72	0	2.80	1.52	4.32	1.84
		Analyses at 96 hr.			
0	96	3.00	0.92	3.92	3.26
84	12	3.13	1.70	4.83	1.84
96	0	3.00	1.63	4.63	1.84

In a further experiment the conditions for agitation just described were reversed, and a standing period preceded that of shaking. Evidence has been presented in another paper to show that rapid removal of the gases increases the fermentation rate (1). It was thought that if agitation followed a period of standing during which the fermentation had become well started, release of the fermentation gases might increase the rate.

The mashes prepared by procedures already described were allowed to stand for 0, 12, 24, 36, 48, 60, 72, 84, and 96 hr., respectively, and at the end of these times were shaken for the remainder of the fermentation periods. Two fermentation periods, 72 and 96 hr., were used, and analyses for diol and ethanol were made at the end of each of these times. All mashes were duplicated.

The results of the experiment are given in Table II. Agitation of the mashes produced a marked inhibition on ethanol and lesser effect on diol formation. There is, however, a slightly beneficial effect on diol formation in mashes shaken for the last 12 to 24 hr. of a 72-hr. fermentation period. The results of the 96-hr. fermentations show a similar effect, which extends to ethanol formation as well.

Aeration with Various Gases

Although the previous experiments involving agitation of mashes showed inhibition of the *A. polymyxa* fermentation, they also demonstrated that aerobic conditions altered the diol–ethanol ratios in favour of the former. It seemed worth while, therefore, to make a more detailed study of this fermentation under aerobic, as well as anaerobic, conditions. For aerobiosis, oxygen and air were used; for anaerobiosis, nitrogen, hydrogen, and carbon dioxide.

These gases were bubbled through 1500-ml. portions of inoculated 15% wheat mash in 3500-ml. Kluyver flasks, which were fitted with "medium" sintered glass disks for gas dispersion. The compressed gases were passed through the mashes at a rate of 333 ml. per minute per litre of mash. Controls consisted of mashes fermented under similar conditions, but without aeration or agitation. The vapours from the mashes were condensed at −70° C. and the condensates added back before sampling for analysis. The samples were withdrawn under sterile conditions at 24, 48, 72, 96, and 120 hr., and analysed for diol, ethanol, acetoin, and, in some cases, total organic acids. The analytical results for each set of conditions are given in Tables III, V, and VI. All data represent means of four separate fermentations.

In general, the method of aeration provided thorough mixing and distribution of the gas. During the first 12 hr. of fermentation the mashes were fairly thick and viscous, and the dispersion of gas was not satisfactory, especially toward the edge of the flask. However, at 24 hr., liquefaction of the mash had reached a stage that permitted distribution of fine bubbles throughout the entire mash. No foaming or frothing difficulties were encountered in the apparatus used.

In Table III yields of diol by C4 (2) and C3 (2) are given. The control for C4 (2) indicates a typical fermentation with the maximum rate occurring in the 24 to 48 hr. period, levelling off at about 72 hr., and proceeding slowly to completion in approximately 120 hr. Under aerobic conditions brought about by oxygen, there was a marked increase in the rate of formation of diol, and the final yield was 13% greater than that of the controls. The air-treated mashes did not have the same accelerated rate of diol formation, although final yields were equal to those of oxygenated mashes. Nitrogen (anaerobic conditions) increased the rate of diol formation more than any other gas but the final yield was less than that of the control. Hydrogen, which is a normal product of the fermentation, increased the rate in the

early stages as compared with that of the control, but not to the same extent as nitrogen. However, final yield of diol was the same at 120 hr. Carbon dioxide, the main gaseous product, increased diol formation only slightly as compared with that of the control, and the final yield was the same as in other anaerobic gases. The results for C3 (2) agreed in general with those of C4 (2), the main difference being the more rapid fermentation rate of the former.

The passage of a vigorous stream of gas through a fermentation mash provides mechanical agitation and facilitates the escape of the fermentation gases. It has already been claimed that the fermentation rate is increased by conditions that provide for rapid removal of carbon dioxide (1). The fact that aeration with carbon dioxide had little effect on the rate lends support to this claim. On the other hand, gases capable of removing carbon dioxide from the mash, e.g., nitrogen, hydrogen, oxygen, and air, all give increased rates of diol formation.

The effects of aerobic and anaerobic conditions on ethanol production are given in Table III also. The control for C4 (2) showed that ethanol

TABLE III

FERMENTATION OF 15% WHOLE WHEAT MASH UNDER AEROBIC AND ANAEROBIC CONDITIONS BY *A. polymyxa*

Organism	Aeration treatment	Butanediol, %					Ethanol, %				
		Time, hr.					Time, hr.				
		24	48	72	96	120	24	48	72	96	120
C3 (2)	Control	0.76	1.85	2.44	2.60	2.70	0.48	1.13	1.55	1.68	1.69
	Air	0.86	1.91	2.68	2.91	2.88	0.44	0.86	1.25	1.33	1.31
	O_2	1.13	2.73	3.06	3.04	3.04	0.42	0.95	1.08	1.10	1.09
	N_2	1.31	2.33	2.44	2.47	2.48	0.86	1.82	1.88	1.87	1.85
	CO_2	0.65	1.93	2.40	2.52	2.59	0.45	1.23	1.73	1.90	1.81
	H_2	0.93	1.99	2.43	2.59	2.62	0.65	1.71	1.86	1.91	1.91
C4 (2)	Control	0.67	1.47	2.18	2.51	2.71	0.40	0.81	1.23	1.51	1.65
	Air	0.61	1.36	2.28	2.90	3.09	0.30	0.55	0.86	1.05	0.98
	O_2	1.02	2.03	2.88	2.95	3.05	0.36	0.56	0.80	0.87	0.94
	N_2	1.10	2.47	2.51	2.49	2.51	0.73	1.76	1.88	1.85	1.83
	CO_2	0.70	1.62	2.35	2.46	2.51	0.45	1.00	1.46	1.62	1.61
	H_2	0.91	2.13	2.51	2.54	2.54	0.56	1.44	1.78	1.90	1.90

formation in a normal fermentation was complete in 120 hr. The effect of aerobic conditions was to decrease the yield of ethanol markedly, the production yields being only slightly over half that of the control. On the other hand, anaerobic conditions as brought about by nitrogen and hydrogen gases caused a marked increase in rate of formation as well as final yield of ethanol. The initial rate with hydrogen is less than that with nitrogen, but final yields were substantially the same. Carbon dioxide caused only a slightly increased rate compared with that of the control. The results with C3 (2) followed,

in general, the pattern of those of C4 (2); the faster over-all rate of fermentation by C3 (2) was again apparent. It is clear from these observations that ethanol formation is retarded by the presence of a hydrogen acceptor (oxygen).

In Table IV the diol–ethanol ratios are given and show more clearly the relation between the products formed during fermentation. The ratio for

TABLE IV

EFFECT OF AEROBIC AND ANAEROBIC CONDITIONS ON THE RELATIVE AMOUNTS OF DIOL AND ETHANOL FORMED IN WHOLE WHEAT FERMENTATION BY *A. polymyxa*

Organism	Aeration treatment	Diol–ethanol ratio				
		Time, hr.				
		24	48	72	96	120
C3 (2)	Control	1.58	1.64	1.57	1.55	1.60
	Air	1.95	2.22	2.14	2.19	2.20
	O_2	2.69	2.87	2.83	2.76	2.79
	N_2	1.52	1.28	1.30	1.32	1.34
	CO_2	1.44	1.57	1.39	1.40	1.43
	H_2	1.43	1.16	1.30	1.35	1.37
C4 (2)	Control	1.67	1.81	1.77	1.66	1.64
	Air	2.03	2.47	2.65	2.76	3.15
	O_2	2.83	3.62	3.60	3.39	3.25
	N_2	1.51	1.40	1.33	1.35	1.37
	CO_2	1.56	1.62	1.61	1.52	1.56
	H_2	1.62	1.48	1.41	1.34	1.34

the control experiment using C4 (2) was fairly consistent, but under aerobic conditions was greatly increased. Its progressive increase with time showed that the production of ethanol is greatly inhibited, while that of diol is actually stimulated. The decrease in ratio in the C4 (2) fermentation at 96 and 120 hr. under nitrogen is explained by a slow increase in the amount of ethanol after that of the diol has become constant. Under anaerobic conditions the ratio, which was approximately 1.5 : 1.0 at 24 hr., dropped to a level of about 1.3 : 1.0 during the 48 to 72 hr. period, when the fermentations were relatively complete, and remained fairly constant thereafter. The ratio in the carbon dioxide treated mashes is intermediate between those of the controls and the anaerobic mashes in the late stages of fermentation.

The effects of the various treatments on acetoin production are shown in Table V. In the controls there is a continual increase to 96 hr., although the amount is not large. Under aerobic conditions there is a marked increase, especially with oxygen. It is interesting to note that the amount is quite small in comparison with that known to occur under similar conditions with *Aerobacter aerogenes* cultures. The fact that the increase in acetoin is not accompanied by a corresponding decrease in diol indicates that it is not being formed by diol oxidation. The accumulation of acetoin is probably due to retardation of its reduction to diol in the presence of excess oxygen.

The amount formed under anaerobic conditions is comparable to that in the control, and tends to become constant after 72 hr.

The 'total products' data recorded in Table V are the sums of diol, ethanol, and acetoin. Organic acid was not included, since it was determined only in certain experiments. Reference to Table VII shows, however, that a

TABLE V

FERMENTATION OF 15% WHOLE WHEAT MASH UNDER AEROBIC AND ANAEROBIC CONDITIONS BY *A. polymyxa*

Organism	Aeration treatment	Acetoin, %					Total products, %				
		Time, hr.					Time, hr.				
		24	48	72	96	120	24	48	72	96	120
C3 (2)	Control	0.03	0.09	0.15	0.14	0.15	1.27	3.07	4.14	4.42	4.54
	Air	0.05	0.15	0.17	0.21	0.29	1.35	2.92	4.10	4.45	4.48
	O_2	0.06	0.12	0.26	0.38	0.41	1.61	3.80	4.40	4.52	4.54
	N_2	0.07	0.16	0.12	0.11	0.12	2.24	4.31	4.42	4.45	4.45
	CO_2	0.03	0.09	0.09	0.11	0.08	1.13	3.25	4.22	4.43	4.48
	H_2	0.04	0.10	0.08	0.08	0.06	1.62	3.80	4.37	4.58	4.59
C4 (2)	Control	0.04	0.05	0.07	0.12	0.12	1.11	2.33	3.48	4.14	4.48
	Air	0.04	0.06	0.09	0.16	0.22	0.95	1.97	3.23	4.11	4.29
	O_2	0.05	0.10	0.22	0.40	0.50	1.43	2.69	3.90	4.22	4.49
	N_2	0.05	0.10	0.12	0.13	0.13	1.88	4.33	4.51	4.47	4.47
	CO_2	0.04	0.07	0.09	0.12	0.10	1.19	2.69	3.90	4.20	4.22
	H_2	0.04	0.07	0.10	0.10	0.10	1.51	3.64	4.39	4.54	4.54

theoretical yield of fermentation products was obtained when the organic acids were included. The 'total products' as calculated in Table V serve as an indication of the over-all rate of the fermentations, as well as the extent of their completeness. Under the conditions of the experiment the control fermentation for C4 (2) was complete in 120 hr., and the total product yield was 4.48%. Under aerobic conditions produced by oxygen, fermentation was complete in 120 hr., and the rate was only slightly greater than that of the control. Air treated mashes also were complete in 120 hr. As the previous discussion on diol–ethanol ratios showed, aerobic conditions increased the rate of formation of diol and inhibited that of ethanol. Anaerobic conditions brought about by nitrogen and hydrogen increased the rates of formation of both diol and ethanol, and significantly shortened the times required for complete fermentation. Carbon dioxide accelerated the rate for the first 48 hr., but the fermentation did not attain completeness in 120 hr.

The concordance of the results for two strains indicates that the effects of aerobic and anaerobic conditions as demonstrated here are common to all *A. polymyxa* strains.

Table VI reports the total organic acid contents of mashes fermented under aerobic and anaerobic conditions by C3 (2) and C4 (2). The main point of interest in comparison with the controls is the marked increase in acid produc-

TABLE VI

ORGANIC ACID PRODUCTION IN AEROBIC AND ANAEROBIC FERMENTATIONS OF WHOLE WHEAT MASH BY *A. polymyxa* AT 120 HR.

Organism	Control		Oxygen		Air		Nitrogen	
	pH	Organic* acids	pH	Organic acids	pH	Organic acids	pH	Organic acids
C4 (2)	5.63	33.60	5.47	90.10	5.49	88.60	5.84	61.60
C3 (2)	5.72	43.70	5.60	85.00	5.58	78.80	5.90	60.04

* *Total organic acids are calculated as cc. N/10 acid per 100 gm. of mash.*

tion under aerobic conditions and a smaller increase under anaerobic conditions. Under anaerobic conditions a higher pH is associated with a relatively high acid content. This suggests that some proteolysis may have taken place and that ammoniacal products were formed that reacted with the acid. This hypothesis is given some support by the observation that the pH of fermented mashes rises on long standing. Furthermore, ammonia has been found in wheat mashes fermented by *A. polymyxa* (1).

The data from the analyses for diol, acetoin, ethanol, and organic acids were recalculated as millimoles of each product formed per 100 millimoles of glucose fermented, and are given in Table VII. In the same table the "glucose

TABLE VII

EFFECT OF AEROBIC AND ANAEROBIC CONDITIONS ON THE DISTRIBUTION AND YIELD OF PRODUCTS IN FERMENTATIONS OF A 15% WHOLE WHEAT MASH BY *A. polymyxa* N.R.C. C3 (2)

* Glucose fermented: 53.2 millimoles per 100 ml.

Product	Millimoles of product per 100 millimoles of glucose fermented			
	Control	Air	Oxygen	Nitrogen
2,3-Butanediol	56.5	60.1	63.5	51.9
Acetoin	3.2	6.2	8.6	2.6
Ethanol	69.1	50.7	44.5	75.2
Organic acids	8.1	14.8	16.0	11.3
Glucose accounted for, %	98.3	99.0	102.3	97.8

* *Glucose calculated on the basis of complete fermentation of a 15% wheat mash; original wheat contained 55% of starch and 3.5% of sugar.*

accounted for" is calculated by assuming that one mole of glucose is required to produce each of the following: 1 mole of diol, 1 mole of acetoin or 2 moles of either ethanol or acid. Since the results show that the "glucose accounted for" always approximated 100%, it can be concluded that the fermentations

were complete, and that the main products had been determined. The data also confirm the observations previously made that under aerobic conditions there were proportional increases in yields of diol and acetoin and a diminution in yield of ethanol as compared with anaerobic conditions.

Fermentation of Glucose by *Aerobacter aerogenes* under Aerobic and Anaerobic Conditions

Since it has been shown that the fermentation rate of *Aerobacillus polymyxa* was markedly increased under anaerobic conditions, it seemed worth while to examine another diol producing organism, *Aerobacter aerogenes*, under similar conditions. As pointed out earlier, it is well known that vigorous aeration of *Aerobacter* cultures is needed to bring about rapid fermentation with a high proportion of diol. From the *Aerobacillus* results it seemed possible that the rate of *Aerobacter* fermentations should be increased under anaerobic conditions, but the diol–ethanol ratio would be much lower.

An experiment was set up to explore these possibilities. The culture medium consisted of 10% of dextrose, 0.09% of potassium monohydrogen phosphate, 0.09% of potassium dihydrogen phosphate, 0.025% of magnesium sulphate, and 0.2% of urea. Six-hundred-millilitre portions of the sterilized media were put in each of three Kluyver flasks, and inoculated with 9 ml. of a 24-hr. culture of *Aerobacter aerogenes*, N.R.R.L No. 199. Air was passed into one flask at the rate of 1 ml. per min. per ml. of mash, nitrogen into another at the same rate, and the third served as an unaerated control. Analyses for diol and ethanol were made at the end of 24 hr. (Table VIII). The fermenta-

TABLE VIII

EFFECT OF AEROBIC AND ANAEROBIC CONDITIONS ON THE FERMENTATION OF GLUCOSE BY *Aerobacter aerogenes* N.R.R.L 199

Aeration treatments	Butanediol, %	Ethanol, %	Total products, %	Ratio
None	0.31	0.21	0.52	1.47
Air	3.01	0.89	3.90	3.35
Nitrogen	2.57	1.47	4.04	1.75

tion rate was greatly increased by the passage of air or nitrogen through the medium. The variation in the diol–ethanol ratio indicated that under vigorous aeration the rate of formation of diol is increased, that of ethanol being depressed; under nitrogen the formation of diol is not accelerated to the same extent, but that of ethanol is greatly increased. The yield of almost equal amounts of total products in 24 hr. showed that the over-all rate of fermentation is approximately the same under aerobic and anaerobic conditions.

A comparison of these results with those obtained with *Aerobacillus* shows a marked similarity in the response of the two organisms to aerobic and anaerobic conditions. The over-all rate of fermentation is a composite of the rates of formation of diol and ethanol, respectively, and is indicated by the time required to reach maximum yields of the two products. In comparison with the controls, the rate of formation of both diol and ethanol is increased under anaerobic conditions by *Aerobacillus* and *Aerobacter* and hence fermentation time is decreased. Under aerobic conditions, diol production by both organisms is stimulated (although to a greater extent by *Aerobacter*), while ethanol formation is markedly inhibited. As a result, in an atmosphere of air or oxygen, fermentation time for *Aerobacter* is greatly decreased while that of *Aerobacillus* is relatively unaffected.

During the preparation of this paper, the author's attention was drawn to a patent issued to Scheffer (3) in which it is claimed that aeration with hydrogen or nitrogen of mashes fermented by bacteria capable of producing 2,3-butylene glycol (2,3-butanediol) increases the fermentation rate. An example with *Aerobacter aerogenes* is given in which fermentation gases freed of carbon dioxide were successfully used for aeration purposes. The results reported in the present paper on the *Aerobacter* fermentation substantiate the aeration claim. The broad claim, unsupported by experimental evidence in the patent, that other bacteria capable of producing 2,3-butanediol would respond in the same manner has been substantiated also by our results with *Aerobacillus polymyxa*.

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PRODUCTION AND PROPERTIES OF 2,3-BUTANEDIOL

VIII. pH CONTROL IN *AEROBACILLUS POLYMYXA* FERMENTATIONS AND ITS EFFECTS ON PRODUCTS AND THEIR RECOVERY[1]

By G. A. Adams[2] and J. D. Leslie[3]

Abstract

Comparative studies have shown that the pH of 15% wheat mashes fermented by *Aerobacillus polymyxa* can be as satisfactorily controlled by ammonium hydroxide as by calcium carbonate. The formation of 2,3-butanediol and ethanol was unaffected by all pH levels tested (5.8, 6.0, 6.5, 7.0) with the possible exception of pH 7.0, where a slight diminution of diol formation appeared at 96 hr. Over the pH range 5.8 to 6.0, the amount of ammonium hydroxide required, the escape of ammonia from the mash, and the production of acid were all minimized. The consumption of ammonia was greatest in the first 36 hr. of the fermentation owing to rapid acid production. Fermentation at the different pH levels did not affect the butanediol–ethanol ratio, which was approximately 1.5.

Replacement of calcium carbonate by ammonium hydroxide reduced the ash content of the unfermented residue from approximately 20 to 4%. Protein contents (N × 5.7) of insoluble residues from carbonate and ammonia treated washes were 25 and 32%, respectively. In both mashes approximately 50% of the unfermented solids were soluble.

Calculation of carbon balances on fermentables showed that increased acid production was accompanied by a decrease in carbon dioxide formation.

Riboflavin and nicotinic acid contents per 100 gm. of fermented mash averaged 19.2 and 1270 μgm., respectively and were unaffected by pH of fermenting mash and heat treatment at 100° C. for 10 hr. The riboflavin showed an 80% increase over that present in the original wheat; nicotinic acid showed a 40% decrease.

Introduction

Carbohydrate dissimilation by *Aerobacillus polymyxa* yields, in addition to diol and ethanol, small amounts of organic acids, which, if left unneutralized, cause the pH to drop to a level of about 5.0. At this level the fermentative process is almost completely inhibited. Previous experiments have shown that addition of 0.5 to 1.0% of calcium carbonate prevents the pH in this fermentation from falling below the range 5.6 to 5.8 (5). Although calcium carbonate is the usual agent for controlling acidity in bacterial fermentations there are several objections to its use in large scale operations with *A. polymyxa*. It is impossible to maintain a uniform pH at a desired level throughout the fermentation period, calcium carbonate increases the ash content in the unfermented residues thereby rendering them less desirable as stock feed, and cooking whole wheat mashes with carbonate at high temperatures has a deleterious effect on the subsequent fermentation. This can be avoided by sterilizing the carbonate separately but such a procedure involves extra equipment and labour.

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[2] *Biochemist, Industrial Utilization Investigations.*

[3] *Chemical Engineer, Industrial Utilization Investigations*

After consideration of other possible agents for controlling pH, ammonia was selected as the most suitable. It overcomes the objections outlined above in that it can be added as required throughout the course of the fermentation, thus permitting the maintenance of any desired pH level. Ammonia gas and its concentrated aqueous solution are both inherently sterile and hence may be introduced directly into fermentation media. From an industrial point of view, ammonia meets the requirements of a plentiful and relatively inexpensive chemical; in addition it can be handled conveniently either as a gas or in aqueous solution, with or without automatic control.

The main purposes of the present investigation were threefold: (1) to compare the relative merits of fermentations the pH levels of which were controlled with ammonia and calcium carbonate, respectively; (2) if ammonia was satisfactory to determine the optimum pH within the range 5.6 to 7.0; and (3) to compare the relative effects of the two agents on the nature, separation, and recovery of the products and by-products.

Since nitrogen is known to be a factor in the nutrition of the organism it might be anticipated that addition of ammonia would affect the fermentation reactions, e.g., rate, completeness, distribution of fermentation products, as well as the distribution of nitrogen in the soluble and insoluble fractions.

Methods and Materials

General

To attain the objectives of the investigation it was necessary to make quantitative measurements of total materials and main components at each step and to calculate material balances; from these balances, losses and distributions could be determined.

Approximately 15% whole wheat mashes were prepared as previously described (5), and 6-litre lots were dispensed into 12-litre flasks. All mashes were inoculated, after cooling, with known amounts of an *A. polymyxa* strain, C3 (2), prepared on 5% whole wheat mash. The fermentation temperature was 30° C. throughout; fermentation times varied from 72 to 120 hr.

For pH control with calcium carbonate 1.0% of the powdered solid was sterilized separately and mixed with the cooked mash prior to inoculation. For control with ammonia the pH of the mash was adjusted at periodic intervals by one of the following methods.

Colorimetric: 10 ml. of alcoholic bromthymol blue was added to each mash, and the pH adjusted by addition, under sterile conditions, of a measured quantity of concentrated ammonium hydroxide (28% ammonia). A sterile sample of the mash containing the indicator and adjusted to pH 6.5 served as a colour reference standard.

Electrometric: A 50 ml. portion of mash was removed from each flask under sterile conditions, and the pH measured and adjusted to the desired level with dilute ammonium hydroxide of known strength. The calculated amount of concentrated ammonium hydroxide was then added to the mash.

In the recovery phases of the work two operations were carried out; filtration of the fermented mashes to separate the insoluble residues from the liquors, and distillation of either the mash or filtrate. Filtration was satisfactorily accomplished through several layers of cheesecloth in a box filter; the filtering area measured 16 by 24 in. Simple distillation of 1- to 2-litre batches was carried out in apparatus of the standard laboratory glassware type using a 3-litre flask and short adapter leading to the condenser; the upper part of the flask and the adapter were lagged to minimize reflux. Distillation was carried to the point where the residues showed definite signs of thermal decomposition, and the distillates were collected fractionally or *in toto* for pH measurement. Batch rectification was done in the same way except that an 18 in. Vigreaux column was added to the apparatus. The column was lagged and reflux was supplied by an air jet at the top.

Analytical

The ground wheat used for mash preparation was analysed for moisture by the A.O.A.C. method (2), nitrogen by a Kjeldahl determination, starch by polarimetry (3), and reducing sugars according to Hanes (4). Solids in the unfermented mashes were measured by evaporating a 50 gm. aliquot and drying the residue to constant weight in an electric oven at 100° C.

The fermented mashes were analysed for diol and ethanol by methods previously described (5). In addition, total solids were measured by the same procedure as described above except that it was necessary to correct the weight of the dried solids for the diol remaining after drying. The latter was estimated in an aqueous extract of the dried solids.

Total acids in the fermented mashes were determined by ether extraction of a 100 ml. portion, at pH 2, for 24 hr. The solvent was removed, the acid extract made up to 100 ml. with water, and an aliquot titrated with $N/10$ sodium hydroxide. No determination of the individual acids was attempted and the results are reported as grams of acetic acid per 100 ml. of mash.

The filtrates were analysed for diol, ethanol, and total solids by the procedures outlined above for mashes; in addition, total nitrogen was determined by the Kjeldahl method. Ammoniacal nitrogen was estimated by making a sample alkaline and aspirating the free ammonia into standard acid.

The residues separated by filtration were thoroughly mixed and 500-gm. portions dried at 100° C. The dried residues, after being corrected for diol as before, were analysed for Kjeldahl and ammoniacal nitrogen, and also for ash by the A.O.A.C. method (2).

Riboflavin and nicotinic acid were estimated by microbiological methods*.

Experimental Results

The first experiments were designed primarily to test the feasibility of controlling a fermentation by addition of ammonia solution in comparison

* *These analyses were kindly performed by W. A. Crandall, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa.*

with the usual method employing 1% calcium carbonate. From general knowledge of bacterial fermentations a pH of 6.5 was arbitrarily chosen for the ammonia controlled mashes.

In Expt. 1 the pH was adjusted at 12-hr. intervals by the colorimetric method. A serious drawback of this procedure was found to be a partial decolorization of the dye by some fermentation product, making pH adjustments unreliable. The fact, however, that the pH at the end of the experiment was approximately 6.6 (by electrometric measurement) indicated that reasonable control had been achieved.

To obtain closer pH control in Expt. 2 the electrometric method was adopted. Although this method permits more accurate control there is a marked drop in pH in the intervals between adjustments, especially during the early stages of the fermentation. The magnitude of these variations is shown in Fig. 1.

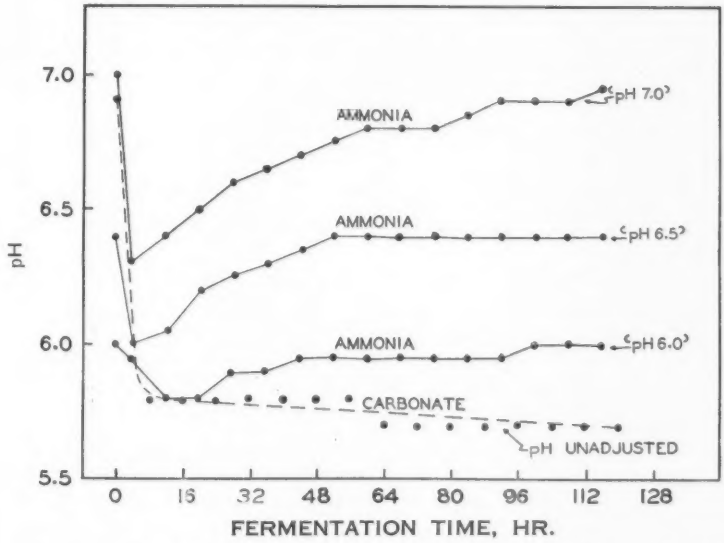

FIG. 1. *Variation of average pH in fermentations controlled with ammonia and with calcium carbonate.*

The fermentation data for the first two experiments are summarized in Table I.

Fermentation efficiencies have been calculated as the ratio of the actual yield of diol plus ethanol to the theoretical yield; the method of calculating the latter, considering starch as the only source of fermentables, has been previously described (5). The results show that in one experiment the efficiency was higher in the carbonate mash than in the ammonia mashes, but that in the other experiment the reverse was true. This leads to the con-

TABLE I

SUMMARY OF DATA ON *A. polymyxa* FERMENTATIONS USING AMMONIA AND CALCIUM CARBONATE FOR pH CONTROL

(Specific gravity of mash, 1.015)

	Expt. 1			Expt. 2		
	Controlled with:			Controlled with:		
	Ammonia		Carbonate‡	Ammonia		Carbonate‡
Weight of original mash, gm.	6500	6555	6595	6335	6394	6223
Wheat in mash, %*	14.30	14.26	13.87	15.16	15.20	15.62
Inoculum, ml.	300	300	300	200	200	200
Ammonium hydroxide (28%) added, ml.	62.0	62.0	—	40.8	42.0	—
Corrected ammonium hydroxide, ml.**	62.0	62.0	—	42.0	43.3	—
Weight of ammonia added per kgm. 15% mash, gm.	2.52	2.51	—	1.65	1.68	—
Weight of mash after fermentation, gm.	6570	6615	6665	5820	5857	6038
Weight of samples removed during fermentation, gm.	0	0	0	406	406	0
Time of fermentation, hr.	74	74	74	93	93	93
Final pH of mash	6.71	6.55	5.60	6.50	6.50	5.70
Diol in fermented mash, %	2.33	2.27	2.41	2.78	2.64	2.64
Ethanol in fermented mash, %†	1.73	1.79	1.80	1.85	1.95	1.60
Diol–ethanol ratio	1.35	1.27	1.34	1.50	1.35	1.65
Fermentation efficiency, %	94.0	94.3	100.1	103.5	102.4	94.0
Total solids in fermented mash, %	4.46	4.40	5.63	4.99	4.92	6.45
Unfermented solids, % Original wheat	31.1	30.8	40.6	32.4	32.3	41.3
Theor. unfermented solids, % Original wheat	32.3	32.3	32.3	32.8	32.8	32.8

‡ *Sixty gm. of carbonate included in weight of mash.*

* *Wheat analyses on "as is" basis: Expt. 1—Moisture, 13.0; starch, 54.7; nitrogen, 2.3%. Expt. 2—Moisture, 13.6; starch, 53.6; nitrogen, 2.1%.*

** *Corrected for sampling.*

† *In Expt. 1, ethanol corrected for indicator solvent.*

clusion that in spite of fortuitous variations there is no essential difference in the fermentation efficiency under the two methods of pH control.

It can also be seen from Table I that the average ratios of diol to ethanol are somewhat different in the two experiments and that within each experiment there are fluctuations from mash to mash. In the light of considerable experience with this fermentation these differences are not considered significant, and are probably due to such uncontrollable factors as the variability of the organism.

The amount of ammonia required per kilogram of 15% mash has been calculated, and it will be noted that this was appreciably greater in Expt. 1

than in Expt. 2. This is attributed to the relatively ineffective method of pH adjustment in the first experiment and is dealt with further in the section on nitrogen balances.

Table I also shows the total solids in the mashes after fermentation, and, allowing for the carbonate, the values agree quite closely with the theoretical amounts as calculated from the analyses of the wheat.

The following sections deal with the comparative effects of ammonium hydroxide and calcium carbonate on the nature, separation, and recovery of the products and by-products. It should be pointed out that only certain components of the fermented mashes, and the steps involved in their recovery, were considered; others such as the recovery of ethanol and the purification of the diol do not constitute problems affected by the factors under study in this work.

Filtration of the fermented mashes permitted estimation of the relative amounts of filtrate and insoluble solids; the analysis of the fractions provided means of calculating the distributions of the various components. Table II summarizes the data and Table III gives the totals and solids balances for this operation.

TABLE II

SUMMARY OF DATA ON FRACTIONS OBTAINED BY FILTRATION OF FERMENTED MASHES

	Expt. 1			Expt. 2		
	A	*B*	*C*	*A*	*B*	*C*
Weight of mash to filter, gm.	6373	6391	6440	5610	5647	5828
Filtrate, total weight, gm.	4257	4367	4310	3660	3707	3793
Diol, %	2.37	2.25	2.43	2.78	2.63	2.73
Solids, %	2.24	2.10	2.43	2.47	2.48	2.97
Kjeldahl nitrogen, %	—	—	—	0.32	0.32	0.20
Ammonia nitrogen, %	—	—	—	0.19	0.19	0.05
Wet solids, total weight, gm.	1700	1665	1780	1720	1770	1715
Dried solids, %	10.52	10.57	13.38	10.34	9.76	13.85
Analysis of dried solids:						
Kjeldahl nitrogen, %	5.55	5.41	4.33	6.10	5.90	4.41
Protein, %, (N × 5.7)	31.6	30.9	24.7	34.8	33.6	25.1
Ash, %	4.53	4.36	18.32	3.46	3.32	25.16

The data in Tables II and III show that the total solids in the fermented mashes were distributed, after filtration, approximately one-third in the filtrates and two-thirds in the filter cakes. This distribution, however, was not truly representative since the filter cakes were not washed. A correction was applied by subtracting from the filter cake solids the weight of the soluble solids in the entrained filtrate. This reduced the average fraction of the total solids in the residues to about 51% and represented true insolubles.

Although the over-all losses in the filtration step may be explained by evaporation, the losses of solids can be attributed only to experimental error.

TABLE III

MATERIAL BALANCES AND DISTRIBUTION OF COMPONENTS IN FILTRATION STEP

	Expt. 1			Expt. 2		
	A	*B*	*C*	*A*	*B*	*C*
Totals						
Fermented mash, gm.	6373	6391	6440	5610	5647	5828
Filtrate, gm.	4257	4367	4310	3660	3707	3793
Wet solids, gm.	1700	1665	1780	1720	1770	1715
Material lost, gm.	416	359	350	230	170	320
Loss, %	6.54	5.62	5.44	4.10	2.81	5.00
Solids						
Solids in fermented mash, gm.	284.1	281.2	362.6	280.5	278.0	376.0
Solids in filtrate, gm.	95.4	91.6	104.7	90.2	92.0	112.6
Solids in residue, gm.	178.6	176.0	238.0	177.8	172.5	237.4
Solids lost, gm.	10.1	13.6	19.9	12.5	13.5	25.8
Loss, %	3.56	4.84	5.49	4.46	4.86	6.86
Fraction of solids in filtrate, %	33.7	32.6	28.9	32.2	33.1	29.9
Fraction of solids in residue, %	62.9	62.6	65.6	63.4	62.1	63.2
Insoluble solids in residue, gm.	143.8	144.0	199.5	139.0	131.8	192.2
Fraction of solids as insolubles, %	50.6	51.2	55.0	49.6	47.4	51.2

It is worth noting that the simple filtration method gave a very wet cake. Although 95% of the recovered filtrate was obtained in two to three hours the cake still contained approximately 90% moisture after 12 hr. filtration.

A comparison of the ammonia and carbonate mashes with respect to the distribution of solids in the filtrates and residues shows no significant differences since the slightly larger amounts of insolubles in the carbonate mashes are due to the presence of excess carbonate. Hence it appears that the use of ammonia does not bring about appreciable solubilization of wheat protein. It is also apparent that the total solids in the carbonate mashes are appreciably greater owing to the added carbonate, and, as Table III shows, this increase is reflected in the filtrate, as well as in the residues. This is evidence that some of the carbonate is converted to soluble compounds during fermentation.

Table II shows that the ash is several times higher in the carbonate than in the ammonia residues. This is not unexpected as it is known that 1.0% of carbonate is considerably in excess of the amount required for neutralization of the acid from a 15% mash. The lower protein percentages in the carbonate residues are, of course, a result of the higher ash contents.

The distribution of nitrogen in Expts. 1 and 2, before fermentation and after filtration, is given in Table IV. The data for Expt. 1 are incomplete, but support those of Expt. 2 and hence have been included. This table reveals several interesting facts, of which probably the most significant is the large loss of nitrogen in Expt. 2. This loss is seen to be relatively greater in

TABLE IV

NITROGEN DISTRIBUTION IN FILTRATES AND RESIDUES

	Expt. 1			Expt. 2		
	A	*B*	*C*	*A*	*B*	*C*
*Kjeldahl nitrogen from wheat in original mash, gm.	21.0	21.0	20.5	18.2	18.4	19.7
Kjeldahl nitrogen from wheat in inoculum, gm.	0.3	0.3	0.3	0.2	0.2	0.2
Nitrogen added as ammonia, gm.	12.5	12.5	0.0	7.8	8.1	0.0
Total nitrogen supplied, gm.	33.8	33.8	20.8	26.2	26.7	19.9
Filtrate:						
Kjeldahl nitrogen, gm.	—	—	—	11.7	11.9	7.6
Ammonia nitrogen, gm.	—	—	—	7.0	7.0	1.9
Residue:						
Kjeldahl nitrogen, gm.	9.9	9.5	10.3	10.8	10.2	10.5
Loss of Kjeldahl nitrogen, gm.	—	—	—	3.7	4.6	1.8
Loss of Kjeldahl nitrogen, %	—	—	—	14.1	17.2	9.0
Fraction of total nitrogen in residue, %	29.3	28.1	49.5	41.2	38.2	52.8
Fraction of wheat nitrogen in residue, %	46.3	44.6	49.5	58.7	54.9	52.8

** All values corrected for sampling.*

the ammonia mashes, *A* and *B*, but is nevertheless appreciable even in the carbonate mash *C*.

Since such losses were not anticipated, nitrogen analyses were not made on the fermented mashes and hence it is not known whether the nitrogen disappeared during fermentation or during the subsequent filtration. It appears most likely, however, that volatile nitrogen compounds were removed during fermentation by the evolved gases, and that such compounds were chiefly ammonia since the losses are substantially higher in the ammonia mashes. The fact that there was a loss in the carbonate mash and that the mash also contained ammonia lends support to this assumption. It is true, of course, that the filtration losses enter into the nitrogen balance, especially as they affect the solids in the residue, but a comparison with Table III shows that the losses there will not account for the nitrogen losses found.

Referring back to the ammonia added to the mashes during fermentation (Table I), it now appears reasonable to attribute the larger amounts of ammonia needed in Expt. 1 to a relatively greater loss. This can be explained by the relatively ineffective method of pH control, which caused the pH to fluctuate considerably above the desired level of 6.5 If ammonia were carried out by the fermentation gases then the losses would obviously be greater at higher pH levels. These observations lead to the conclusion that the theoret-

ical amount of ammonia required per kilogram of 15% mash is considerably less than 1.65 gm. The practical significance of this is discussed in a later section of this paper.

Another point illustrated by the data of Table IV is that only about half of the original wheat nitrogen appears in the solids of the residue. The rest was solubilized and, as the carbonate mash shows, partly converted to ammonia.

It should be pointed out that the omission of washing of the filter cakes affected the distribution of nitrogen between filtrates and residues. A correction for the nitrogen in the entrained filtrate can be applied similarly to that mentioned above for the distribution of solids. This will decrease the nitrogen fractions in the residues by approximately 20% of the values shown in Table IV.

Simple distillation of the filtrates at atmospheric pressure enabled an estimate to be made of the quantity of syrup remaining after removal of most of the aqueous phase. This syrup results from accumulation of the soluble solids in the filtrate. Since it is susceptible to thermal decomposition when its solid content approaches 50% it is impossible to remove the remaining liquid which contains a high percentage of the total diol. Since the handling of this syrup is a difficult technical problem it is desirable to reduce the volume to a minimum.

The results of the distillation step are shown in Table V. It is apparent from the results that there was no appreciable difference in the amounts of syrup formed in the filtrates from the ammonia and carbonate treated mashes, nor were any physical differences observed in the various syrups. Furthermore, this lack of significant variation under the two treatments is reflected in the distribution of diol between the distillates and syrups. The differences shown in the data may be attributed to lack of uniform conditions, e.g., a slight amount of reflux occurring in the distilling head, and variable initation of decomposition owing to point heating of the flask.

TABLE V

DISTRIBUTION OF COMPONENTS BY DISTILLATION OF FILTRATES

	Expt. 1			Expt. 2		
	A	*B*	*C*	*A*	*B*	*C*
Weight of filtrate distilled, gm.	2400	1900	2127	2107	1921	1930
Weight of distillate, gm.	2265	1815	2035	1970	1811	1790
Weight of syrup, gm.	148	82	91	127	101	118
Weight of material lost, gm.	−13	3	1	10	9	22
$\frac{\text{Weight of syrup}}{\text{Weight of filtrate}}$, %	6.2	4.3	4.3	6.0	5.2	6.1
Solids in syrup, %	33.3	47.1	51.0	41.2	48.1	45.2
Fraction of original diol in syrup, %	55.2	60.0	55.3	64.5	72.5	68.5

Fermentations at Different pH Levels

It has been shown in Expts. 1 and 2 that the pH of whole wheat mashes fermented with *A. polymyxa* may be satisfactorily controlled by addition of ammonia. Since the use of ammonia permitted adjustment of the pH to any desired level it became of interest to compare fermentations carried out at various pH's within the range 5.6 to 7.0.

Six-litre quantities of 15% mash were prepared, inoculated, and fermented as previously described. The wheat used in this experiment had the following analysis: moisture, 13.2; starch, 55.1; nitrogen, 2.3; protein, 13.1% (N × 5.7). Duplicate mashes were fermented at each of three pH levels: 6.0, 6.5, and 7.0; pH adjustment by the electrometric method was made at eight-hour intervals. Duplicate mashes containing 1.0% of calcium carbonate served as controls, and at the end of each 24 hr. period samples were taken from all mashes for chemical analyses.

Table VI shows the amounts of diol and ethanol formed at various times in the fermenting mashes. For purposes of comparison all quantities have been calculated on the basis of a 15% mash, and the tabulated results are the means of the duplicates.

TABLE VI

COMPARATIVE EFFECTS ON PRODUCTS YIELD OF CONTROLLING pH WITH AMMONIA AND WITH CALCIUM CARBONATE

Time, hr.	—	Ammonia			1% Calcium carbonate
		pH = 6.0	pH = 6.5	pH = 7.0	
24	Diol, %	0.63	0.46	0.61	0.83
	Ethanol, %	0.40	0.31	0.43	0.54
48	Diol, %	1.93	1.71	1.83	1.94
	Ethanol, %	1.15	1.12	1.12	1.19
72	Diol, %	2.54	2.57	2.55	2.61
	Ethanol, %	1.66	1.67	1.65	1.66
96	Diol, %	2.73	2.75	2.62	2.75
	Ethanol, %	1.75	1.73	1.68	1.75
120	Diol, %	2.75	2.79	2.66	2.80
	Ethanol, %	1.81	1.77	1.80	1.84

It will be seen from the data of Table VI that the fermentation rates of mashes controlled at the various pH levels by ammonia do not differ appreciably either among themselves or from the rate in the carbonate controlled mash. A possible exception may be the decreased rate of diol formation at pH 7.0 after 72 hr.

The fermentation efficiencies and diol–ethanol ratios, for the various pH levels, are tabulated in Table VII. The ratio was remarkably constant not only at the different pH levels but also at the various fermentation times,

TABLE VII

THE COMPARATIVE EFFECTS ON DIOL–ETHANOL RATIO AND ON FERMENTATION EFFICIENCY OF CONTROLLING pH WITH AMMONIA AND WITH CALCIUM CARBONATE

pH	Time in hours							
	Diol–ethanol ratio					Fermentation efficiency, %		
	24	48	72	96	120	72	96	120
5.8 (carbonate)	1.55	1.62	1.58	1.57	1.52	93.8	99.4	102.2
6.0	1.57	1.67	1.53	1.55	1.52	92.8	98.6	100.4
6.5	1.52	1.52	1.53	1.59	1.57	93.7	98.8	100.4
7.0	1.43	1.64	1.54	1.56	1.48	92.5	94.9	98.4

although the lower ratio at pH 7.0 and 120 hr. may be significant. The relatively greater variation in ratios at the 24 and 48 hr. periods seems to be an inherent characteristic of this fermentation. However, the actual level of the ratio in this experiment is of little significance since it has been shown that it is governed largely by the degree of aerobiosis (1).

The fermentation efficiencies were calculated from 72 hr. on since only at this time were the fermentations sufficiently complete to be of practical interest. These results show that at the various levels the fermentations approached completeness at approximately the same rate. As previously mentioned, the fermentation at pH 7.0 may be an exception. In general, it may be concluded from these data along with those of Expts. 1 and 2 that variation in the pH in the range 5.6 to 7.0 has little effect on the fermentation efficiency.

As previously pointed out, the method of pH control did not achieve as precise regulation as was desired. In Fig. 1 the mean pH values for each eight-hour period are plotted, and for comparison the course of the pH in the mash containing calcium carbonate is included. The relatively large fluctuations in the early stages of the fermentations indicated the formation of surprisingly large amounts of acid. More effective control would have necessitated more frequent sampling; this would have increased the dangers of contamination and was undesirable from the standpoint of depleting the mash volumes. It is felt, however, that with the possible exception of the mashes at pH 7.0 sufficient control was established to permit valid assessment of the effect of the different pH levels. As previously shown in Table VII, the mashes that were intended to be at pH 7.0 showed a decreased rate of fermentation after 72 hr. Since Fig. 1 shows that only during this period did the pH approximate the desired level it is reasonable to suppose that had a pH of 7.0 been maintained throughout, the over-all rate of fermentation would have been correspondingly decreased; in addition, larger amounts of acid might have been expected.

In Fig. 2 are plotted the mean volumes of 1.48 *N* ammonium hydroxide solution required to adjust 50-ml. samples of the mashes to the prescribed pH levels at the end of each eight-hour period. These quantities are an indication of the acid formed in each period. Two significant facts are shown by these

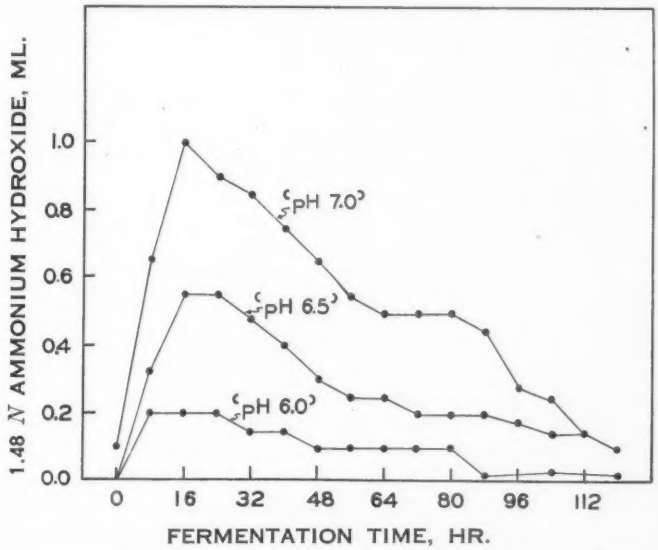

FIG. 2. *Ammonium hydroxide required to adjust 50-ml. aliquots of mash to prescribed pH levels during fermentation.*

curves. First, the production of acid was greatest during the first 24 hr. of fermentation, thus confirming the conclusion made from Fig. 1; and secondly the higher the pH level the greater was the amount of acid produced. The latter observation was proved by measuring the total acids in the fermented mashes at 120 hr. The results, calculated as acetic acid, are given in Table VIII.

TABLE VIII

THE COMPARATIVE EFFECTS ON ORGANIC ACID YIELD OF CONTROLLING pH WITH AMMONIA AND WITH CALCIUM CARBONATE

Neutralizing agent	pH	Acetic acid, %	Acetic acid, moles per litre of fermented mash
Carbonate	5.8	0.242	0.0403
Ammonia	6.0	0.294	0.0490
Ammonia	6.5	0.385	0.0642
Ammonia	7.0	0.667	0.1112

Materials Balance

Table IX gives the materials balance for the fermentations of Expt. 3. The weights of dry gases given off during the fermentations were calculated by subtracting the true weights of the fermented mashes from the total weights of the original mashes plus inoculum plus the concentrated ammonium hydroxide added. The true weight of each fermented mash was arrived at

TABLE IX

SUMMARY OF DATA ON FERMENTATIONS AT DIFFERENT pH LEVELS

(All data are the means of duplicates)

Neutralizing agents	Carbonate	Ammonia		
pH of mash	5.8	6.0	6.5	7.0
Initial weight of mash, gm.	5770*	5827	5790	5050
Wheat in mash, %	15.31	15.41	15.56	15.63
Weight of inoculum, gm.	203	203	203	203
Actual weight of fermented mash, gm.	4330	4348	4350	3641
**True weight of fermented mash, gm.	5743	5765	5775	5124
Solids in actual fermented mash, %	6.25	4.86	4.39	4.70
†Weight of ammonium hydroxide added, gm.	0.00	16.64	45.52	75.09
†Weight of ammonia added per kgm. of 15% original mash, gm.	0.00	0.78	2.12	3.99
†Moles of ammonia added per litre of fermented mash	0.000	0.0476	0.1302	0.2414
Weight of dry gas evolved, gm.	290	282	267	204
Weight of dry gas per kgm. of 15% mash, gm.	49.2	47.1	44.5	38.7
Kjeldahl nitrogen in actual fermented mash, %	0.367	0.406	0.475	0.568
Ammoniacal nitrogen in actual fermented mash, %	0.011	0.030	0.098	0.166

* *Sixty gm. of calcium carbonate not included.*

** *See text for meaning of true weight.*

† *Corrected for mash sampling.*

by correcting for the following: samples removed, gases and ammonium hydroxide associated with samples, water vapour removed by the fermentation gases, and finally the ammonia lost during the fermentations.

It will be noted from Table IX that the weight of evolved gases decreases with increasing pH, and this suggests a correlation with the increase in total acids shown in Table VIII. The nature of this relation is made clear by the carbon balance in Table X. The close agreement between the total amounts of carbon before and after fermentation indicates that all the main products were satisfactorily accounted for.

It will be noted that, excepting the carbonate mash, the apparent loss in carbon increases with the pH. This is due probably to two causes. The acids were calculated as acetic, but it is known from previous experiments that a portion of the total acid is lactic. Hence the average carbon per mole

TABLE X

CARBON BALANCES FOR FERMENTATIONS AT DIFFERENT pH LEVELS

Basis: 37.2 gm. of carbon from 1 kgm. of 15% wheat mash*

Neutralizing agent	Carbonate	Ammonia		
pH of mash	5.8	6.0	6.5	7.0
Weight of carbon in:				
**Diol, gm.	14.41	14.05	14.38	13.90
Ethanol, gm.	9.28	9.05	8.94	9.26
† Totals acids, gm.	0.94	1.11	1.45	2.55
Carbon dioxide, gm.	13.16	12.60	11.91	10.33
Total carbon accounted for, gm.	37.79	36.81	36.68	36.04
Loss in carbon, gm.	−0.59	0.39	0.52	1.16
Loss in carbon, %	−1.61	1.05	1.40	3.12

* *Includes carbon from inoculum starch. Assumed that starch was only source of fermentables and was totally fermented.*

** *Includes acetoin.*

† *Calculated as acetic acid.*

of acid would be greater than that calculated and the error from this factor would be greater with greater amounts of acid. Again, the dissolved carbon dioxide remaining in the mashes at the end of fermentation was not determined. This, too, would be greater at higher pH levels. The slight excess of carbon found in the carbonate mash may be attributed to a partial release of the carbon dioxide from the calcium carbonate.

Since the diol plus ethanol yields are relatively constant at the different pH levels it must be concluded that the increase in acid at the higher pH levels occurs at the expense of decreased carbon dioxide production. This is noteworthy since it is known that in a normal *A. polymyxa* fermentation (controlled with calcium carbonate) almost 50% of the glucose is converted to carbon dioxide. Hence, it appears that without appreciably altering the yield of diol plus ethanol or the over-all rate of fermentation the chemical balance of the system may be shifted by variation of pH.

From the data in Table IX it was possible to make nitrogen balances on the four fermentations. These are summarized in Table XI, and show a progressive loss in nitrogen (as ammonia) with increasing pH. Losses of this order were previously found in Expt. 2, but at that time they could not be definitely attributed to the fermentation step. The present evidence shows that the theoretical amounts of ammonia required per kilogram of 15% mash are less than those actually found. In fact, the data in Tables IX and XI show that approximately twice as much ammonia was added as remained fixed in the mashes after fermentation. It would be expected that the amounts of ammonia required to maintain the pH of the mash at the prescribed levels would be almost chemically equivalent to the acids formed.

TABLE XI

NITROGEN BALANCE FOR FERMENTATIONS AT DIFFERENT pH LEVELS*

Neutralizing agent	Carbonate	Ammonia		
pH of mash	5.8	6.0	6.5	7.0
Kjeldahl nitrogen in original mash, gm.	20.35	20.65	20.71	18.17
Kjeldahl nitrogen in inoculum, gm.	0.23	0.23	0.23	0.23
Ammonia nitrogen added, gm.	0.00	3.84	10.50	17.32
Total nitrogen supplied, gm.	20.58	24.72	31.44	35.72
Kjeldahl nitrogen in fermented mash, gm.	21.05	23.40	27.40	29.10
Ammonia nitrogen in fermented mash, gm.	0.63	1.73	5.65	8.52
Non-ammonia nitrogen in fermented mash, gm.	20.42	21.67	21.75	20.58
Ammonia in fermented mash, moles/litre	0.0080	0.0218	0.0711	0.1203
Loss in ammonia nitrogen, gm.	−0.63	2.11	4.85	8.80
Loss in non-ammonia nitrogen, gm.	0.16	−0.79	−0.81	−2.18

* *All values corrected for sampling.*

A comparison of the data in Tables VIII and XI reveals that this assumption is justified.

As previously observed in Expt. 2 a small amount of ammonia was found in the carbonate controlled mash. This undoubtedly arose from proteolytic breakdown of the gluten fraction. The apparent increases in the non-ammoniacal nitrogen contents of the mashes controlled with ammonia suggest that some of the added ammonia was converted to other nitrogen compounds; further investigation is required to prove this point.

Vitamin Contents of Fermented Mashes

Although it was not considered likely that the pH of the fermenting mashes would affect their vitamin contents it seemed desirable to supplement the material balances already made with estimates of those vitamins most likely to be unaffected by the recovery process. Such vitamins are important if the unfermented solids are to be recovered as feed.

Attention was restricted to riboflavin and nicotinic acid, both of which are relatively thermostable. Since these vitamins are water soluble, determinations were made on the unfiltered mashes of Expt. 3.

The ultimate recovery of both the soluble and insoluble feed fractions involves heat treatments and it is important therefore to know the stability of the vitamins under such conditions. This was tested by slow evaporation of the whole mashes at atmospheric pressure, under reflux. The evaporations took about 10 hours and reduced the volumes by approximately 75%; the temperature of each mash ranged from 99° to 105° C. The residues were then made up to their original volumes and reanalysed for riboflavin and

nicotinic acid. The results are given in Table XII and include, for comparison, the corresponding contents of the original wheat.

TABLE XII

RIBOFLAVIN AND NICOTINIC ACID CONTENTS OF MASHES BEFORE AND AFTER FERMENTATION AND AFTER HEAT TREATMENT

Basis: 100 gm. of fermented mash

Neutralizing agent	pH of mash	Original wheat		Fermented mash		Heat treated mash	
		Riboflavin	Nicotinic acid	Riboflavin	Nicotinic acid	Riboflavin	Nicotinic acid
Carbonate	5.8	19.0	1250	30.4	706	28.6	710
Ammonia	6.0	19.4	1280	39.8	815	39.0	780
Ammonia	6.5	19.4	1280	32.3	785	30.1	764
Ammonia	7.0	19.2	1265	34.3	755	33.7	776
Means	—	19.25	1269	34.2	765	32.9	758

All values expressed as μgm.

The data show that the heat treatment caused no significant losses of either vitamin. In the fermentation process itself, however, an 80% increase in riboflavin and a 40% decrease in nictotinic acid are indicated. Since the total solids remaining after fermentation (on a dry basis) are roughly one-third of the original wheat (on an "as is" basis) it follows that the concentrations of these vitamins in the dried solids would be approximately 5.4 and 1.8 times, respectively, their concentrations in the wheat. The amounts of the above vitamins in the yeast extract of the inoculum were not great enough to affect significantly the results. Little significance is attached to the variations between mashes at different pH levels.

Application to Large Scale Process

The laboratory investigations described in this paper provide several results, which, it is felt, will prove applicable to the large scale process.

It has been shown feasible to carry out the fermentation using ammonia as a means of controlling pH, and for several reasons the range of pH 5.8 to 6.0 is most favourable. At this lower level the amount of ammonia required, the escape of ammonia from the mash, and the production of acid are all minimized. It might be pointed out that although the increased acid production at higher pH levels means a more efficient utilization of the starch, such an advantage would be more than offset by the difficulties of recovering the products.

It is obvious from Fig. 1 that automatic injection of the ammonia will be necessary to maintain the pH closer to the prescribed level, and hence the amounts of ammonia required per unit of mash will be somewhat greater

than those calculated in Table IX. On the other hand, the losses of ammonia could probably be reduced by introducing it below the level of the mash.

If a figure of 1.0 gm. per kgm. of 15% mash is taken as the ammonia requirement at pH 6.0 a simple calculation shows that the amount per bushel of wheat (60 lb.) fermented will be a 0.40 lb. The corresponding amount of calcium carbonate (as 1.0% in the mash) is 4.0 lb. At 20c. per lb. for anhydrous ammonia and 4c. per lb. for calcium carbonate the costs per bushel of wheat would be 8 and 16c., respectively. It has been shown recently that the fermentation will proceed satisfactorily with 0.5% of carbonate, and on this basis the costs of the two chemicals become equal. In addition to the above the use of ammonia will avoid the handling and mixing of a powdered solid.

A further advantage in the use of ammonia is the production of solid residues of very low ash content as compared with the residues from carbonate mashes. Since the nitrogen content is unimpaired these residues should have greater values as feed.

Acknowledgment

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PRODUCTION AND PROPERTIES OF 2,3-BUTANEDIOL

IX. THE EFFECT OF VARIOUS NUTRIENT MATERIALS ON THE FERMENTATION OF STARCH BY *AEROBACILLUS POLYMYXA*[1]

By Sybil B. Fratkin[2] and G. A. Adams[2]

Abstract

Wheat starch is a poor medium for fermentation by *Aerobacillus polymyxa*. The solubles recovered from the separation of starch and gluten in patent flour enhance the fermentation but not as effectively as the similar fraction from whole wheat flour. Addition of supplements is necessary for a satisfactory yield of products in a reasonable length of time. Wheat gluten has no stimulatory effect but bran and shorts are both effective, the latter being slightly superior. An 8% starch medium fortified with the solubles from whole wheat required a 2.5% supplement of shorts to bring fermentation by *A. polymyxa* to 90% completeness in 72 hr.

Of the various supplements tested, a 1% addition of malt sprouts proved to be the most effective, fermentation being 90% complete in 72 hr. Shorts, bran, Cerogras (dehydrated young oats), alfalfa, soya beans, yeast extract, and corn-steep liquor follow in order of decreasing effectiveness.

The solubles from whole wheat when ashed have no beneficial effects on the fermentation of starch by *A. polymyxa*.

Introduction

During the course of investigations on the production of 2,3-butanediol by fermentation of whole wheat with *Aerobacillus polymyxa*, it was found that the recovery of diol by distillation was hindered as evaporation progressed by accumulation of soluble solids in the form of a syrup. It has been shown that a large proportion of the soluble solids originate from solubilized wheat protein (1). It seemed advantageous, therefore, to remove the bulk of the wheat protein (gluten) prior to the fermentation provided its absence did not seriously interfere with the efficiency of the fermentation. Preliminary evidence already existed that most of the gluten could be removed without adversely affecting the fermentation (6). A satisfactory process for the separation of starch and gluten from patent flour had been developed in these laboratories (8). When used with whole wheat flour, this process removed the bran and shorts with the gluten and left a starch slurry containing soluble fractions of the wheat. From preliminary experiments it was apparent that the starch + the water soluble fraction of wheat ("wash water") was not a satisfactory fermentation medium for *A. polymyxa* and that it was necessary to fortify this substrate with additional supplements.

Recent researches in bacterial nutrition have established the importance of certain accessory substances for growth as well as fermentation. Growth factors for *A. polymyxa* have not been worked out completely as yet, but

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Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as Paper No. 32 on the Industrial Utilization of Wastes and Surpluses and as N.R.C No. 1359.

[2] *Biochemist, Industrial Utilization Investigations.*

biotin has been shown to be essential, whereas thiamin, which stimulated growth of some strains, inhibited others. Pantothenic acid, nicotinic acid, riboflavin, pyridoxine, and inositol were ineffective as nutrilites (5). Yeast extract as a nutrient source for *A. polymyxa* has been reported previously (4, 6) but for large scale operations use of either pure nutrient materials or yeast extract is not practicable and a cheaper source of nutrients is desirable.

A number of cheaper supplements have been used with success in other fermentations. Tatum, Peterson, and Fred (11) used cabbage, oranges, yams, potatoes, alfalfa, soya beans, wheat middlings, and malt sprouts to increase the production of butyl alcohol in the fermentation of corn-mash by certain butyric acid bacteria. Corn-steep liquor was used to supply necessary nutrients for *Acetobacter suboxydans* (10) and *Lactobacillus delbrückii* (9). Recently Fulmer, Bantz, and Underkofler (3) reported use of alfalfa extract as a nutrient supplement for growth and chemical activity of *A. suboxydans*.

The purpose of the present investigation was to find suitable supplements for a starch fermentation by *Aerobacillus polymyxa*.

Materials and Methods

Three types of starch mashes were used; starch–whole-wheat-wash-water, starch–patent-flour-wash-water, and commercial-wheat-starch–tap-water. The process for separating starch and gluten developed in these laboratories (8) was applied to whole wheat and patent flours. The final concentration of starch was adjusted to 8.0 to 8.25% so that the yields could be compared with those from 15% whole wheat mashes. The starch suspensions were preliquefied for 20 min. at 70° C. with 1% barley malt (on the basis of the weight of wheat that would contain the starch present), and cooked at 100° C. for five minutes. Three hundred millilitres of mash was dispensed into 500 ml. Erlenmeyer flasks containing calcium carbonate (3 gm.) and the nutrient supplement being tested. After sterilization for one hour at 15 lb. pressure, followed by cooling to 30° C., 10 ml. of a 24 hr. culture was added. For all experiments reported here a locally isolated strain of *A. polymyxa* designated as N.R.C. 3 (2) was used. All fermentations were carried out at 30° C.

In most experiments, fermentations were allowed to proceed for 48, 72, 96, and 120 hr. A separate flask was set up for each time interval so as to avoid introducing contaminants during sampling.

Methods of analysis for butanediol and alcohol have been described in a previous paper (6).

Experimental Results

Effect of Gluten on Fermentation of Wheat by Aerobacillus polymyxa

Gluten was washed out by hand (2) from a sample of whole wheat flour, drum dried, and milled to 60 to 80 mesh. The gluten was added back in varying percentages of the original amount present to a mash made of the residual starch, bran, and wash water. Results of fermentation are given in Fig. 1. Only curves for total products are shown since the diol–ethanol ratio

was not affected by different amounts of gluten. It is apparent that gluten had very little effect on the yield of product. A small amount (10 to 25%) did increase the yield slightly in the early stages but this effect disappeared as fermentation neared completion. Controls of whole wheat mash always

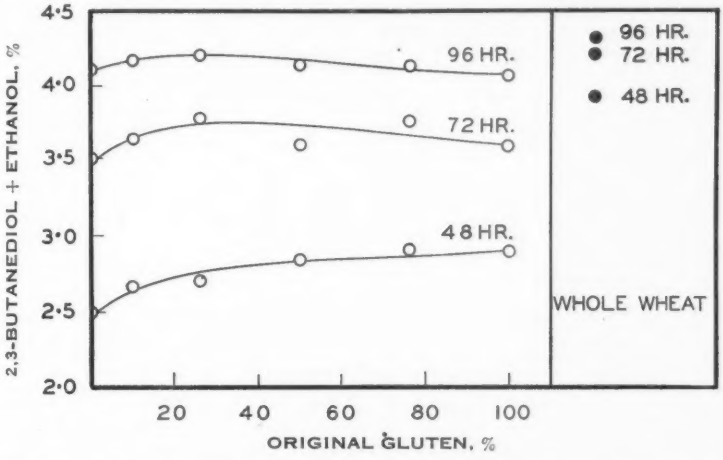

FIG. 1. *Effect on fermentation of adding gluten to a medium of starch, bran, and wash water in comparison with a whole wheat medium of the same starch content.*

gave slightly higher yield of product than even the best of the reconstituted mashes, but this is explicable on the basis of a slightly lower starch content of the latter. In addition, the rate of fermentation in whole wheat mashes was much greater than in the reconstituted mashes containing all the original gluten.

Effect of Bran, Shorts, and Wheat Germ in Fermentation of Starch

Since whole wheat mashes were fermented satisfactorily, it seemed desirable to assess the values of the bran, shorts, and wheat germ fractions as nutrient supplements in a starch fermentation. The effect of each fraction was determined on the three types of starch mashes previously described.

While the bran and shorts fraction of wheat is subject to considerable variation in quantity, it may be regarded as 27% of the wheat. The relative amount of each depends largely on the milling process but is usually equal. Thus in 300 ml. of a 15% whole wheat mash (8.25% of starch), there would be 12.15 gm. of bran + shorts, i.e., approximately 4% of the weight of the mash. Wheat germ content of whole wheat is 1 to 2% and in a 15% wheat mash there would be approximately 0.3 gm. per 100 ml. of mash.

Preliminary experiments showed that bran, shorts, and wheat germ were very similar in their stimulatory action on the fermentation of starch by *A. polymyxa*. However, addition of all the wheat germ originally present in the wheat was no more effective than the same amount of bran or shorts.

TABLE I

EFFECT OF WHEAT GERM, BRAN, AND SHORTS AS NUTRIENT SUPPLEMENTS ON FERMENTATION OF STARCH

Nutrient	%	2,3-Butanediol + ethanol, %			
		48 hr.	72 hr.	96 hr.	120 hr.
Control		1.16	1.52	1.84	2.11
Wheat germ	0.1	1.23	1.73	1.94	2.41
Bran	0.1	1.30	1.66	2.06	2.48
Shorts	0.1	1.18	1.68	2.00	2.53
Control		1.16	1.52	1.84	2.11
Wheat germ	0.3	1.31	1.66	2.31	2.72
Bran	0.5	1.64	2.22	2.45	3.06
Shorts	0.5	1.58	2.14	2.66	2.76

Hence, use of wheat germ for commercial production would not be feasible because of its higher cost. A typical result, showing the interchangeability of bran, shorts, and wheat germ as supplements is given in Table I.

Since it would be more practical to add either all bran or all shorts in a large scale operation, amounts up to and including the total amount present in a 15% whole wheat mash of both bran and shorts were used. These amounts were equivalent to 4% of the weight of the mash. An experiment was done in which both bran and shorts were added in varying proportions ranging from all bran to all shorts. The results showed that shorts was slightly superior to bran and that there was nothing specific in the action of either, but rather that their stimulatory effects were additive (Table II).

TABLE II

EFFECT OF BRAN + SHORTS IN VARYING PROPORTIONS ON FERMENTATION OF STARCH

Supplement*	2,3-Butanediol + ethanol, %			
	48 hr.	72 hr.	96 hr.	120 hr.
100% Bran	2.50	4.01	4.14	4.24
75% Bran + 25% shorts	2.60	3.99	4.21	4.29
50% Bran + 50% shorts	2.51	4.07	4.23	4.20
25% Bran + 75% shorts	2.72	4.16	4.18	4.19
100% Shorts	2.93	4.10	4.21	4.28

**A supplement of 4% of the weight of the mash was used in each case.*

(*1*) *Mash, Starch–Whole-wheat-wash-water*—Effects of bran and shorts in a mash fermentation on starch mixed with the wash water from whole wheat are shown in Fig. 2. Comparison of the two sets of curves shows that shorts gave slightly higher total products than bran but this effect can be explained partially on the basis of higher starch content of shorts (shorts 11.32%, bran

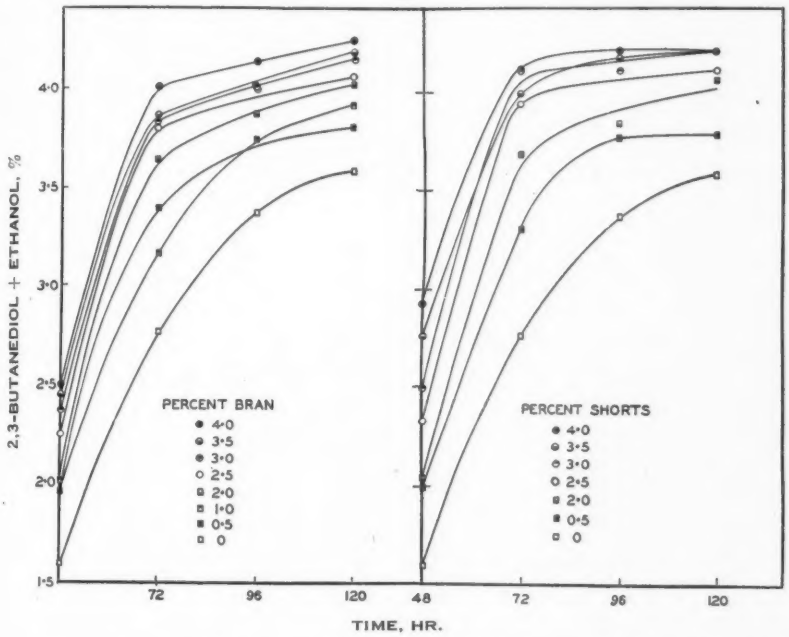

FIG. 2. *Effect of various amounts of bran and shorts on fermentation of a starch–whole-wheat-wash-water mash.*

7.88%). With either supplement, the rate of fermentation increased with increase in nutrient added. This effect was more noticeable at the lower concentrations where a slight increase in supplement caused a marked increase in rate. However, a saturation point for supplement is reached where further amounts gave only slightly higher yields, which were due principally to the additional starch supplied by the supplement. It follows, therefore, that for this mash, 2.5% of either bran or shorts was the optimum concentration.

Using 2.5% of bran and shorts, fermentations were 88 and 90% complete, respectively, at 72 hr. These percentages were calculated on the basis of a 7.64% starch content of the mash and corrections were applied for the starch in the nutrient supplement. It is interesting to note that starch and wash water alone fermented fairly well but the fermentation did not reach completion even after 144 hr. This indicates that the wash water either did not contain some essential factor or was lacking in sufficient quantities of the necessary nutrients to carry fermentation to completion.

(*2*) *Mash, Starch–Patent-flour-wash-water*—Fig. 3 shows the effects of bran and shorts in a starch–patent-flour-wash-water mash. In this experiment also, shorts gave an over-all higher yield of products than bran. A comparison with Fig. 2 shows that the rate of fermentation was much slower for this mash than in the whole-wheat-wash-water–starch mash since the curves

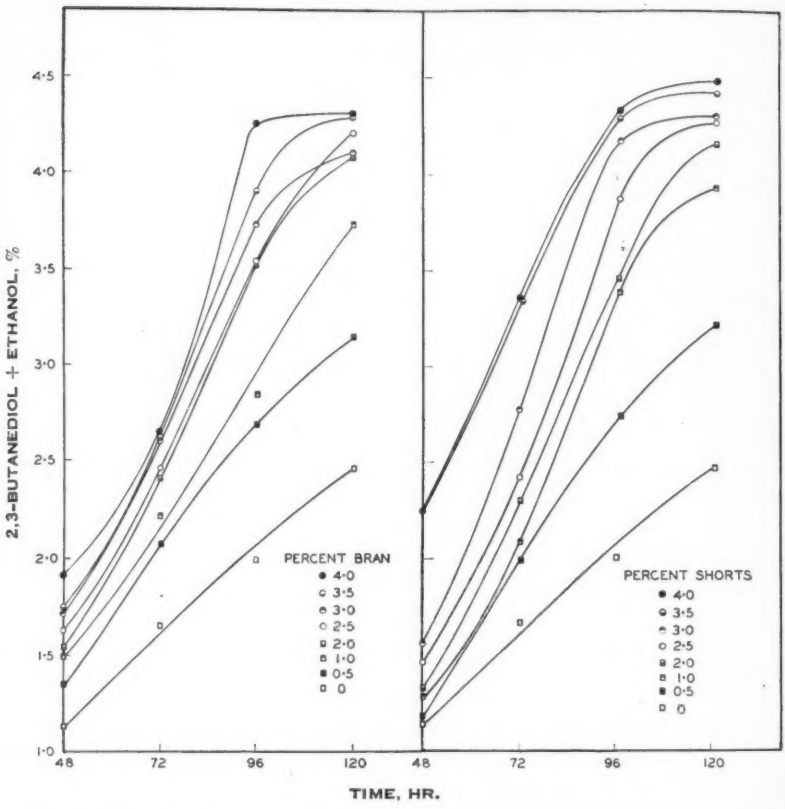

FIG. 3. *Effect of various amounts of bran and shorts on fermentation of a starch–patent-flour-wash-water mash.*

in the former do not begin to flatten out till 96 hr. In addition, at least a 3% supplement was required to bring the fermentation to completion. With a 3% supplement of bran and shorts, fermentations at 96 hr. were 81 and 89% complete, respectively. The starch and wash water alone was fermented rather poorly and at 120 hr. fermentation was only 55% complete.

(*3*) *Mash, Commercial-starch–Tap-water*—Effects of bran and shorts on the fermentation of a mash of commercial starch and tap water are shown in Fig. 4. Again shorts increased the rate of fermentation slightly more than bran. The control mashes of starch and tap water were fermented very poorly and at 120 hr. fermentations were only 25% complete. Addition of small amounts of supplement was markedly beneficial but a 3 to 3.5% supplement of either bran or shorts was necessary for optimum results. With 3% of either supplement fermentations were 88% complete at 96 hr.

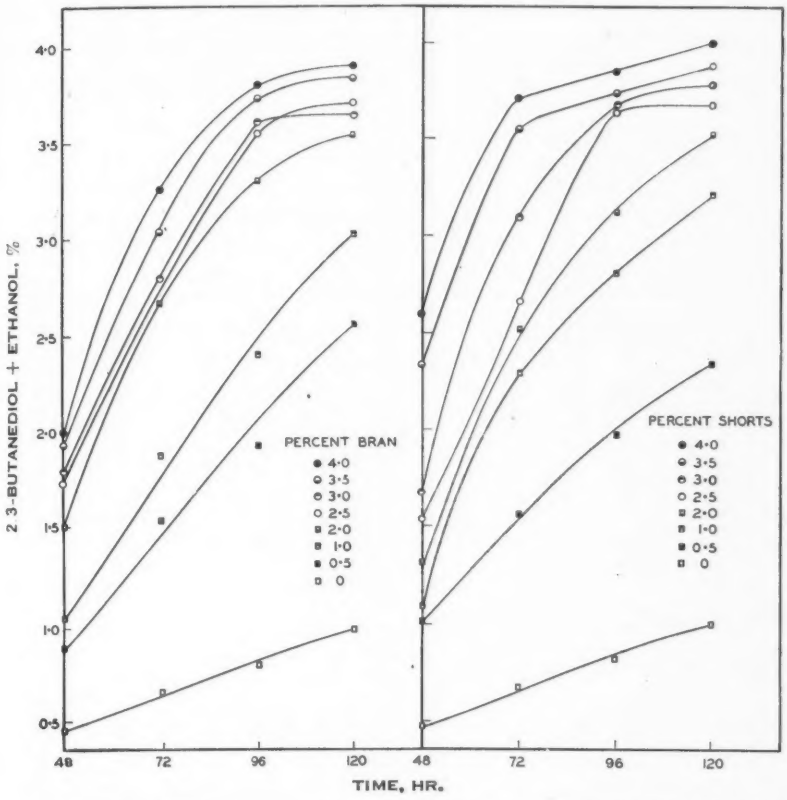

FIG. 4. *Effect of various amounts of bran and shorts on fermentation of a starch–tap-water mash.*

Effect of Other Supplements

In addition to bran, shorts, and wheat germ a number of other substances in varying concentrations were used as nutrient supplements in a starch–whole-wheat–wash-water mash. These included yeast extract, corn-steep liquor, malt sprouts, unsterilized malt sprouts, alfalfa, soya beans, and Cerogras*. For comparison, bran and shorts were also included in the series. The results given in Table III were corrected for fermentables in the added nutrients.

Of the various supplements tested, malt sprouts gave the best results, fermentation being 90% complete in 72 hr. with only 1% of this supplement. Unsterilized malt sprouts have been successfully used as a supplement for *Lactobacillus delbrückii* in a lactic acid fermentation (7). Unsterilized malt sprouts in the *Aerobacillus polymyxa* fermentation gave even better results than sterilized sprouts, but the danger of introducing contamination more

* *Cerogras—dehydrated young oat plants.*

TABLE III

EFFECT OF VARIOUS SUPPLEMENTS IN VARYING CONCENTRATIONS IN A STARCH–WHOLE-WHEAT–WASH-WATER MASH* FERMENTATION

Nutrient	%	2,3-Butanediol + ethanol, %		
		48 hr.	72 hr.	96 hr.
Control		1.61	2.99	3.52
Malt sprouts**				
Sterilized	1.0	2.95	4.03	4.21
Unsterilized	0.5	3.43	0.76†	0.80†
Shorts‡	1.0	2.01	3.64	4.11
Bran§	1.0	2.19	3.57	4.16
Cerograss††	1.0	2.64	3.61	4.03
Alfalfa	1.0	2.49	3.87	4.06
Soya beans	1.0	1.48	3.13	3.80
Yeast extract	0.5	2.72	3.75	3.89
Corn-steep liquor	0.5	2.09	3.13	3.54
Malt sprouts				
Sterilized	2.0	3.14	4.25	4.45
Unsterilized	1.0	3.61	4.24	4.11†
Shorts	2.0	1.95	3.80	4.23
Bran	2.0	2.03	3.68	4.22
Cerograss	2.0	2.85	3.85	4.18
Alfalfa	2.0	2.94	3.77	4.01
Soya beans	2.0	2.03	3.58	4.12
Yeast extract	1.0	2.88	3.75	3.96
Corn-steep liquor	1.0	2.47	3.51	3.80
Malt sprouts				
Sterilized	3.0	3.44	4.36	4.48
Unsterilized	1.5	3.37	2.40†	2.39†
Shorts	3.0	2.24	4.11	4.38
Bran	3.0	2.05	3.54	4.22
Cerograss	3.0	2.90	3.87	4.17
Alfalfa	3.0	3.17	4.15	4.21
Soya beans	3.0	1.99	3.64	4.06
Yeast extract	1.5	2.87	3.58	3.95
Corn-steep liquor	1.5	1.93	3.16	3.57

* *Starch content of mash, 7.93%.*

** *Fermentables in malt sprouts, 22.59%.*

† *Contaminated.*

‡ *Starch content of shorts, 11.32%.*

§ *Starch content of bran, 7.88%.*

†† *Cerograss—dehydrated young oat plants; obtained from Greenmelk Co., Ltd., Wallaceburg, Ont.*

than outweighed the beneficial effects. In several experiments an attempt was made to overcome possible contaminants by addition of large amounts of inoculum (three or five times the amount usually employed), but again increase in yield did not compensate for the additional inoculum required. Next in order of effectiveness were shorts and bran, both of which, in a 3% concentration gave a fermentation approximately 90% complete in 72 hr. When used in their highest concentration, Cerograss, alfalfa, soya beans, yeast extract, and corn steep liquor followed in order of decreasing effectiveness.

Effect of Ash of Wash Water

Wash water obtained from a whole wheat flour by the starch separation process was taken to dryness on a steam-bath and then ashed in a furnace at 550° C. to constant weight. The ash was then taken up in its original volume of tap water and slightly acidified with sulphuric acid. Mashes were prepared with the appropriate amounts of starch and fermented in the usual manner. Control mashes of starch–tap-water and starch–whole-wheat-wash-water were fermented at the same time. Results given in Table IV showed that ashing destroyed the growth promoting factors (4).

TABLE IV

EFFECT OF WHOLE WHEAT WASH WATER AND ITS ASH IN FERMENTATION OF STARCH

Description of mash	2,3-Butanediol + ethanol, %		
	48 hr.	72 hr.	96 hr.
Starch + ash + tap water	0.54	0.69	0.86
Starch + tap water	0.63	0.90	0.98
Starch + whole wheat wash water	1.73	2.32	3.01

Discussion

From the experimental results it is apparent that with suitable nutrient supplements *A. polymyxa* can ferment 8% starch mashes satisfactorily. It was not the purpose of this investigation to fractionate out any specific growth factor or factors but rather to find a readily available supplement containing the necessary factors to bring about a satisfactory fermentation of starch. Whole wheat wash water which itself supported growth to some extent had no growth promoting effect when ashed. This suggests that the growth factors are organic in nature but does not exclude the possible need for certain inorganic salts as well.

A starch–whole-wheat-wash-water mash with a supplement of 1% of malt sprouts or 2.5% of shorts appears to be the best material for commercial production. In normal times, malt sprouts are as cheap as shorts and as readily available. A starch–patent-flour-wash-water mash with a slight increase in supplement and time of fermentation might be also used as a fermentation medium. In this instance the by-product from the separation process would be a relatively pure gluten, which has a much higher market value than the gluten-bran feed from a whole wheat flour separation. The question as to whether whole wheat or patent flour could be most profitably used is largely an economic one.

Acknowledgments

The authors wish to thank Mr. A. L. Shewfelt for his assistance in carrying out the starch separations from whole wheat and patent flours used in the course of this work.

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DRIED WHOLE EGG POWDER

XVIII. THE KEEPING QUALITY OF ACIDULATED, GAS-PACKED POWDERS OF LOW MOISTURE CONTENT[1]

By Jesse A. Pearce[2], Margaret Reid[2], and W. H. Cook[3]

Abstract

Acidification of liquid egg prior to drying did not improve subsequent storage life, although pH measurements showed that powder from untreated egg became acid more rapidly during storage. Reduction in the moisture content (total volatiles) from 4.7 to 3.0% doubled, and reduction from 4.7 to 1.7% tripled, the storage life of dried whole egg powder as assessed by fluorescence tests. The maximum storage life predicted for the low moisture powder by this test was only 36 wk. at 27° C. and 5 wk. at 38° C. Palatability tests suggested that the product was somewhat less perishable, as a powder of 1.7% moisture was considered fit for use as an egg dish after 64 wk. at 27° C. Gas-packing low moisture powders in an atmosphere of carbon dioxide appeared to be slightly more effective as a means of retaining palatability than packing in an atmosphere of air or nitrogen, but was particularly effective in preventing loss of solubility (assessed by potassium chloride values) during storage.

Introduction

During an investigation of the effect of added substances on the keeping quality of egg powder, it was observed that fluorescence development in powders containing either citric or lactic acid was more rapid than in control powder (3). Contrary indications had been observed elsewhere (4).

Accelerated tests, done in these laboratories, showed that the rate of deterioration of egg powder increased with increase in moisture content; therefore, it was recommended that, to maintain quality during storage and transport, dried egg should have a moisture content of not more than 5% and probably 2% or less (7). It was further noted that reduction to 1.4% had marked preservative action, although some deterioration occurred when powders were held at 37° and 48° C. (6).

A study of the effect on keeping quality of packing in nitrogen, carbon dioxide, *in vacuo*, and in the form of compressed tablets showed that only carbon dioxide had a beneficial effect (8). Continued investigation of the effect of carbon dioxide showed that this method of packing afforded some protection against heat deterioration, particularly on the solubility of the powder (6).

At the request of the Advisory Committee to the United States Army Quartermaster Corps, experiments were undertaken in co-operation with American research organizations to verify the advisability of combining those features believed to result in powders of better keeping quality, e.g.,

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[2] *Biochemist, Food Investigations.*

[3] *Director, Division of Applied Biology.*

acidifying liquid egg prior to drying, drying egg to low moisture content, and packing in carbon dioxide or nitrogen or both. The present paper deals with the storage behaviour of such specially treated powders, both at a relatively high temperature, 38° C. (100° F.), and a temperature likely to be met during ordinary handling of dried egg powders, 27° C. (80° F.).

Methods

The quality tests applied were: the evaluation of palatability by a taste panel of six persons scoring on a basis of 10 to 0, 10 being the equivalent of excellent fresh egg (1); a fluorescence measurement of an extract of defatted egg powder in 10% sodium chloride solution (2) and a measurement of solubility in a 10% potassium chloride solution (5). Measurements of pH were made on samples as reconstituted for cooking. Moisture content was determined as total volatiles by a modification of the standard A.O.A.C. vacuum oven procedure. The technique of this modification has been previously described (5).

Materials

The egg powders used in this investigation were prepared in a Canadian egg drying plant from frozen egg. One portion was dried by current methods, resulting in a product having a moisture and volatile content of 4.7%; another portion was dried to have the lowest moisture and volatiles feasible in a single stage commercial operation (3.0%); some of this powder was subjected to a second, combined cooling and vacuum drying process, which further reduced the moisture and volatile content to 1.7%. A further sample of liquid egg was acidified to a pH of 6.7 before subjection to drying and subsequent redrying to produce a low volatile powder (1.7%). These powders were stored in tin plate containers, the headspace gas being air.

Samples of the acidified and untreated products were also packed in tin plate in atmospheres of carbon dioxide and nitrogen. The carbon dioxide content of the headspace gas was approximately 100, 75, 50, 25, and 0%; the remainder of the gas was nitrogen except for a trace of oxygen.

Powders stored at 38° C. (100° F.) were sampled after 1, 2, 4, 8, and 16 weeks' storage, while powders stored at 27° C. (80° F.) were sampled after 4, 8, 16, 32, and 64 wk.

Results

The effect of moisture content is shown in Fig. 1, while effects of acidifying liquid egg and of using atmospheres of carbon dioxide and nitrogen, either alone or mixed, are given in Tables I and II and Fig. 2. Only the mean values for each variable, averaged over all other conditions, are shown in Tables I and II, since this was a convenient method of summarizing the data. The inert atmosphere in the headspace of the tins is recorded on an "as-packed" basis. Measurement after the one, two, and four weeks at 38° C.

and after four and eight weeks at 27° C. showed that the pressure inside the container had been reduced by about $\frac{1}{3}$ atm. and that the carbon dioxide content of the headspace gas was 90, 45, 5, 2, and 0%, the remainder of the gas being nitrogen and about 0.5% oxygen. Sorption of carbon dioxide by the powder was believed responsible for this phenomenon and is under investigation.

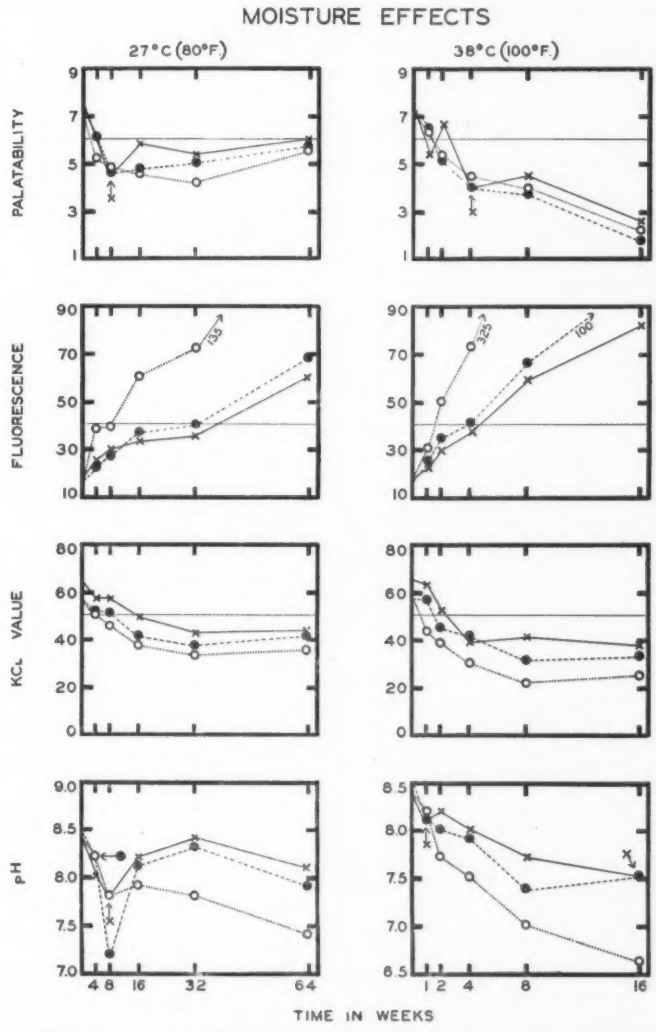

FIG. 1. *The effect of moisture levels on the keeping quality of dried whole egg powder stored at 27° C. (80° F.) and 37° C. (100° F.). Light lines show limits of desirability as an egg dish. 4.7% moisture, ○; 3.0% moisture, ●; 1.7% moisture, ×.*

TABLE I

TABLES OF MEANS AND ANALYSES OF VARIANCE OF TESTS ON EGG POWDER OF 1.7% MOISTURE CONTENT PREPARED FROM LIQUID EGG WITH AND WITHOUT ADDED ACID AND STORED IN ATMOSPHERES OF CARBON DIOXIDE AND NITROGEN AT 38° C. (100° F.)

Tables of means

Variable under study	Palatability	Fluorescence value	Potassium chloride value	pH
Powder				
From acidified liquid egg	5.6	38.6	55.5	7.3
From untreated liquid egg	5.6	36.5	53.9	8.0
Approximate gas composition[1]				
100% carbon dioxide	5.7	35.2	57.6	7.6
75% carbon dioxide	5.8	35.5	56.4	7.6
50% carbon dioxide	5.6	38.0	53.8	7.7
25% carbon dioxide	5.7	39.0	53.2	7.7
0% carbon dioxide	5.3	40.3	52.4	7.7
Storage time				
Initial	7.7	17.1	63.1	7.9
1 week	6.8	21.1	64.8	7.8
2 weeks	6.2	27.3	61.4	7.8
4 weeks	4.7	33.2	50.7	7.6
8 weeks	4.5	50.1	47.8	7.4
16 weeks	3.6	76.9	40.4	7.6

Analyses of variance

Variance attributable to:	Degrees of freedom	Mean square			
		Palatability	Fluorescence value	Potassium chloride value	pH
Powder (plain vs. acid)	1	0.00	66 **	35 *	7.9 **
Gas composition	4	0.40	58 **	61 **	0.026
Storage time	5	24.2 **	5035 **	968 **	0.452*
Powder × gas composition	4	0.06	5.6*	11	0.022
Powder × storage time	5	0.21	67 **	17 *	0.060*
Storage time × gas composition	20	0.35*	11.4**	7.3	0.026
Residual	20	0.15	2.0	4.8	0.020

[1] *Remainder of gas, nitrogen.*

* *Exceeds the 5% level of statistical significance.*

** *Exceeds the 1% level of statistical significance.*

Acid Powder

Acidifying liquid egg prior to drying appeared to afford little protection to the powder (Tables I and II). The only measurement showing significant differences between powders at both storage temperatures was, of course, pH. At 38° C., in addition to difference in initial pH, the powder prepared from untreated egg tended to become acid more rapidly than powder prepared from

TABLE II

TABLES OF MEANS AND ANALYSES OF VARIANCE OF TESTS ON EGG POWDER OF 1.7% MOISTURE CONTENT PREPARED FROM LIQUID EGG WITH AND WITHOUT ADDED ACID AND STORED IN ATMOSPHERES OF CARBON DIOXIDE AND NITROGEN AT 27° C. (80° F.)

Tables of means

Variable under study	Palatability	Fluorescence value	Potassium chloride value	pH
Powder				
From acidified liquid egg	6.2	27.6	57.0	7.4
From untreated liquid egg	6.3	28.4	56.8	8.2
Approximate gas composition[1]				
100% carbon dioxide	6.3	27.2	59.3	7.8
75% carbon dioxide	6.4	26.4	58.2	7.8
50% carbon dioxide	5.9	27.7	56.6	7.8
25% carbon dioxide	6.3	27.8	55.6	7.8
0% carbon dioxide	6.0	30.9	54.8	7.8
Storage time				
Initial	7.7	17.1	63.1	7.9
4 weeks	5.4	22.2	60.5	7.8
8 weeks	5.6	23.2	62.4	7.6
16 weeks	5.7	29.5	55.8	7.8
32 weeks	6.5	29.4	51.7	8.1
64 weeks	6.3	46.6	47.9	7.6

Analyses of variance

Variance attributable to:	Degrees of freedom	Mean square			
		Palatability	Fluorescence value	Potassium chloride value	pH
Powder (plain vs. acid)	1	0.00	11	1.01	8.4 **
Gas composition	4	0.45	36 **	41 **	0.26 **
Storage time	5	7.4 **	1057 **	385 **	0.385**
Powder × gas composition	4	0.81	6.7	8.8	0.016
Powder × storage time	5	0.36	1.0	8.4	0.014
Storage time × gas composition	20	0.24	5.4	7.5	0.016*
Residual	20	0.32	3.7	5.3	0.006

[1] *Remainder of gas, nitrogen.*

* *Exceeds 5% level of statistical significance.*

** *Exceeds 1% of statistical significance.*

acidified liquid egg. While it is difficult to explain this phenomenon, it is believed of little practical importance since acidification of liquid egg prior to drying had no other significant effect.

Effect of Moisture Content

There is evidence from Fig. 1 that reduction in moisture content resulted in a slower rate of decrease in palatability during storage. Powder with a moisture content of 1.7% was about one palatability unit better than that

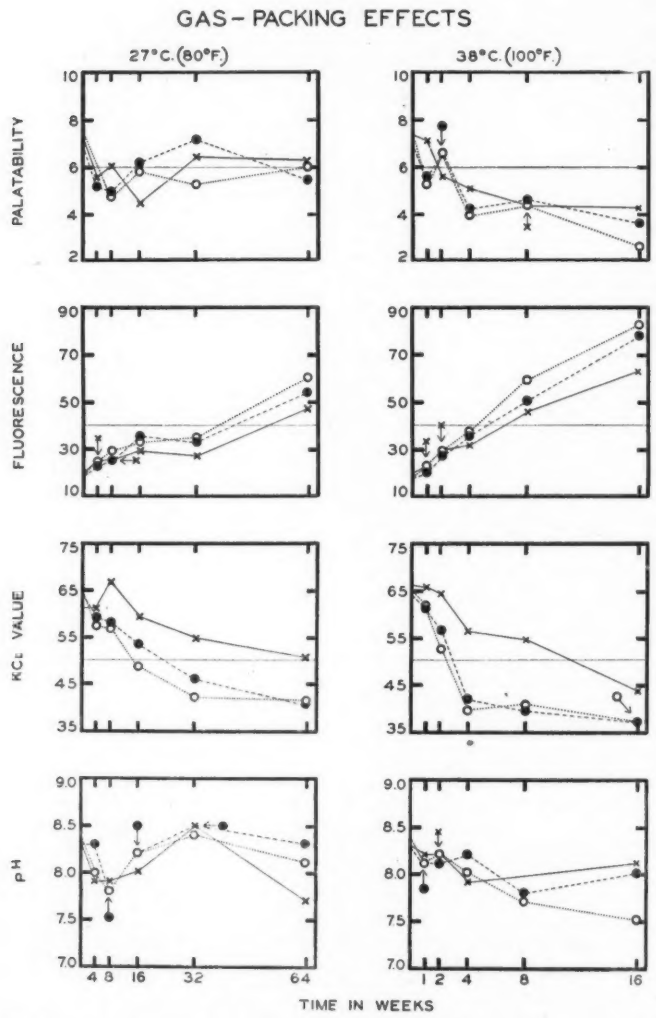

FIG. 2. *The effect of gas-packing on the keeping quality of dried whole egg powder (moisture and volatiles 1.7%) stored at 27° C. (80° F.) and 37° C. (100° F.). Light lines show limits of desirability as an egg dish. Air, ○; nitrogen, ●; carbon dioxide, ×.*

with 4.7% moisture after storage for 16 wk. at 38° C. and about half a palatability unit better after 64 wk. at 27° C. Powders stored at 27° C. for eight weeks had exceptionally low palatability and pH values; nevertheless, the 1.7% moisture powder was still considered suitable as an egg dish after 64 weeks' storage in an atmosphere of air at 27° C.

It had been noted that fluorescence values of 40 are about the equivalent of a palatability score of 6, the limit of desirability of the powder as an egg dish (1). Using this criterion, it was observed that the reduction in moisture to 3% doubled, while further reduction to 1.7%, tripled the storage life at 38° C. (Fig. 1). However, even this extended life was only five weeks. At 27° C., powders having 4.7% moisture had a storage life of only about six weeks, while powders of 3.0 and 1.7% moisture withstood storage of 32 and 36 wk., respectively.

Measurements of potassium chloride value and pH, both related to palatability (5), also indicated an increase in storage life as volatile content was reduced.

Packing in Carbon Dioxide and Nitrogen

The effects of gas-packing egg powder of 1.7% volatile content in atmospheres of air, carbon dioxide, and nitrogen are shown in Fig. 2. The values depicted here contrast data from Fig. 1 with data from Tables I and II. Only the data for air, nitrogen, and carbon dioxide packing are shown since these showed most clearly the differences between methods of packing. Again palatability scores were variable, but there was some indication of increased storage life as a result of gas-packing, carbon dioxide being more effective than nitrogen, which was in turn more effective than air. Fluorescence and pH measurements also indicated that carbon dioxide was more effective than nitrogen in increasing storage life. The carbon dioxide packed material showed a marked drop in pH after 64 wk. at 27° C., thus explaining the significant differential effect noted in Table II. This may be the result of some reaction between carbon dioxide and egg powder to produce more highly acidic products.

Generally, slight improvement in palatability resulted from increasing the carbon dioxide content in the headspace gas (Tables I and II). Both fluorescence and potassium chloride values indicated that improvement resulted from increased carbon dioxide content. This improvement became more noticeable as storage progressed.

The most pronounced effect was evident in the improved solubility of the powders packed in an atmosphere of carbon dioxide: after eight weeks' storage at 38° C., powder in an atmosphere of carbon dioxide had a potassium chloride value of 55, while powders packed in air or nitrogen had a value of about 38. After 64 wk. at 27° C. the carbon dioxide packed material still had a solubility greater than 50.

Acknowledgments

The authors wish to thank Mr. D. B. W. Reid for making the statistical computations.

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THE EFFECT OF WHEAT GERM OIL ANTIOXIDANTS AND NATURAL REDUCING SUBSTANCES ON THE STABILITY OF WHOLE MILK POWDER[1]

By R. A. Chapman[2] and W. D. McFarlane[3]

Abstract

Storage trials have been conducted on a large number of roller and spray-process whole milk powders. Accelerated tests at 65° C. were found to give an accurate indication of the relative keeping qualities of the samples. Wheat germ oil antioxidants were found effective in inhibiting deterioration due to copper. Reducing substances that develop in milk powders during storage in a moist atmosphere or at elevated temperatures are strong antioxidants and may offset the effect of added antioxidants. The riboflavin content of several powders with a high concentration of reducing groups decreased appreciably during storage.

Introduction

Tallowy odours and flavours produced by the action of atmospheric oxygen on the butterfat are considered to be one of the most important forms of spoilage in whole milk powder (13). Copper and iron contamination, due to the solvent action of heated milk on metallic surfaces during processing, is an important factor in the development of this defect. In recent years a number of investigations have been conducted in an effort to retard oxidative rancidity by the use of antioxidants.

Peters and Musher (17) claimed that the fresh flavour of powdered milk was retained when oat flour was added to liquid milk prior to spray-drying and when the oat flour was mixed with the dry powder. Waite (19) has used oat flour and hydroquinone with some success. However, the latter substance, at a level of 0.5% of the weight of the fat, imparted an objectionable metallic flavour to the milk powder. Jack and Henderson (9) have employed Avenex No. 7, Avenol, and ascorbic acid for this purpose. Avenex at a level of 2 to 3% of the milk solids, or 0.2% of Avenol, prevented the development of oxidized flavour for approximately eight months. Gum guaiac and hydroquinone were the most effective of the antioxidants employed by Hollender and Tracy (7), and ethyl gallate, at levels of 0.01 and 0.03% of the liquid milk, has been reported by Hilditch (6) to treble the life of spray-dried whole milk powders.

Lips and McFarlane (15) found that wheat germ oil fortified with citric acid was an effective antioxidant in the prevention of oxidative rancidity in lard and shortening. Since this combination is non-toxic and both constituents occur naturally in foods, it seemed desirable to investigate its use

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[2] *Assistant Professor of Chemistry.*

[3] *Professor of Chemistry.*

as an antioxidant in whole milk powder. A similar study by Tracy *et al.* (18), started at the same time as this investigation, has now been reported. They found that wheat germ oil was most effective at a level of 0.2% of the butter fat and the inhibiting effect was reinforced by citric acid.

Several workers (3, 11) have found that sulphydryl compounds, which developed in liquid milk heated to high temperatures, were responsible for the inhibition of oxidized flavours. Hollender and Tracy (7) reported that powders prepared from milk heated to 170° F. for 30 min. prior to drying was less likely to develop an oxidized flavour than those samples prepared from milk heated to 150° or 190° F. The presence of sulphydryl groups has been suggested by Jack and Henderson (9) as the reason for the improved stability of the product prepared from milk preheated to high temperatures. In view of these facts it appeared desirable to determine accurately the effect of these reducing groups on the stability of the butterfat in whole milk powders, especially in relation to the action of wheat germ oil antioxidants.

Early in this study it became evident that an accelerated storage test was an important requirement. Acceleration by light, heat, and metallic catalysts has been employed in the study of oxidative rancidity in fats and oils. Recently, however, Hilditch (6) has questioned the validity of all accelerated tests and heat-accelerated tests in particular. Heat-accelerated tests on milk powders have been discussed by Lea, Moran, and Smith (13) who found that temperatures above 60° C. rapidly produced caramel-like flavours and discoloration, while similar defects were obtained more slowly at 37° and 47° C. In this investigation these off-flavours masked the detection of oxidized flavours by organoleptic means, but had no apparent effect on the formation of peroxides.

Materials and Methods

A large number of milk powder samples containing the following antioxidants were prepared by commercial firms.

Formula *A*—Straight wheat germ oil.*

Formula *B*—Wheat germ oil plus 1% of citric acid.

Formula *C*—Wheat germ oil plus 2% of citric acid.

Formula *D*—65% wheat germ oil, 30% of soya-bean lecithin, 5% of citric acid.

Formulas *B* and *C* were prepared by dissolving citric acid monohydrate in absolute ethyl alcohol, thoroughly mixing the solution into the required amount of wheat germ oil, and agitating the mixture in a Waring Blendor for five minutes. The alcohol was then removed *in vacuo*. Formula *D* was prepared by the method outlined by Lips†.

It was noted that the peroxide value of wheat germ oil was frequently as high as 60 milliequivalents per kilogram. It was believed that if the peroxide

* *The wheat germ oil employed throughout this investigation was the solvent extracted product prepared by Viobin Corp., Monticello, Ill., U.S.A.*

† *A. Lips, Ph.D. thesis, McGill University, Montreal, Que. 1944.*

value of the oil were reduced, its stabilizing value would be enhanced. Therefore, the antioxidants were heated at 150° C. for 60 min. under vacuum in the presence of a nickel catalyst, since previous experiments had indicated that this treatment would remove the peroxides.

The treated antioxidants were then tested by the Swift stability test as modified by Lips and McFarlane (15). However, the results indicated that the treatment had not improved the stabilizing action of the oils. Vitamin E determinations by the method of Parker and McFarlane (16) revealed that the heat treatment had not reduced the tocopherol content.

The copper content of all the samples was determined by the method of Kerr (12) with minor modifications, which included the addition of a few drops of cresol red to indicate the presence of slight excess of ammonia before the addition of the carbamate reagent, and extraction of the coloured complex with carbon tetrachloride instead of chloroform. Solubility determinations were made according to the directions of Howat and associates (8). The milk powders were reconstituted at 60° C. as recommended by Lea, Moran, and Smith (13), cooled to 20° C., and centrifuged at 4000 r.p.m. The supernatant liquid was decanted into an evaporating dish and dried *in vacuo* at 100° C. for five hours.

Peroxides were determined by the method of Chapman and McFarlane (1). The procedure was found quite satisfactory but occasionally a reagent was prepared that developed a considerable amount of colour. This difficulty could be minimized if all the ammonium thiocyanate were dissolved before the addition of the anhydrous acetone (14). The solution was then thoroughly mixed before adding the ferrous ammonium sulphate. It was also necessary to purify the acetone carefully. Reducing groups were estimated by the method of Chapman and McFarlane (2).

Discussion of Experimental Data

Series I—Storage at Various Temperatures

In the early stages of the investigation, the milk powders were stored at room temperature and 37° C., but the deterioration was often relatively slow. It therefore was necessary to accelerate the oxidation of many samples by storing them at higher temperatures. An experiment was conducted to compare the stability of milk powders at room temperature and at elevated temperatures.

Two spray-dried samples were prepared, one of which contained Antioxidant *D* at a level of 0.1% of the butterfat. They were stored in open containers at room temperature, 37°, 55°, and 65° C. Peroxide values were determined at intervals with the results shown in Fig. 1.

The conclusion as to the relative stability of the two powders would be the same irrespective of whether the data obtained at 65° C. or at room temperature was considered. However, at 55° and 65° C. the samples developed caramel-like flavours, which tended to mask the tallowiness of the

butterfat as determined organoleptically. For our purpose the advantage of reducing the storage time from 150 to 15 days outweighed the disadvantage of not being able to test the quality of the sample by taste. The effect of the antioxidant was evident in all cases and the relatively poor keeping quality of the powder can probably be attributed to its high copper content (8.79 p.p.m.).

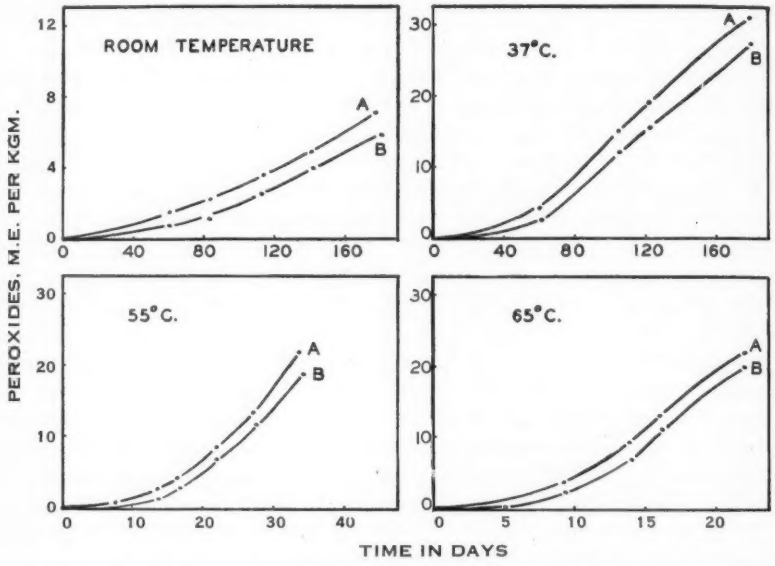

FIG. 1. *The stability of spray-process whole milk powder as influenced by the storage temperature. Sample A—control; Sample B—treated with antioxidant D.*

The results of another experiment, carried out to determine the effect of light in addition to heat, indicated that although light accelerated the oxidation of the butterfat, elaborate precautions would be necessary to ensure uniform exposure of the samples. Heat alone seemed more satisfactory as the basis of an accelerated test.

Series II—Storage at Room Temperature

Two samples of spray-dried whole milk powders were prepared with commercial equipment, and one was treated with Antioxidant *D* at a level of 0.1% of the butterfat. They were stored at room temperature in friction-top cans and sampled periodically. The results of the peroxide tests are shown in Fig. 2. Both powders were relatively unstable and this can be attributed chiefly to copper contamination, analyses showing 23.5 p.p.m. It is clear that under such conditions wheat germ oil *D* is a potent inhibitor of the catalytic action of copper.

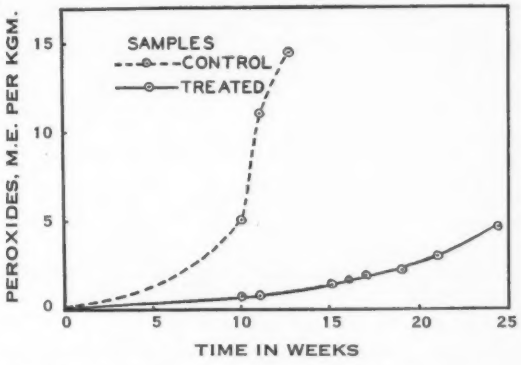

FIG. 2. *The effect of a wheat germ oil antioxidant on the stability of spray-process whole milk powder stored at room temperature.*

Series III

(*a*) *Storage of Fresh Samples at 65° C.*

A series of eight spray-dried powders, containing antioxidants *A*, *C*, and *D* at levels of 0.1 and 0.05% of the butterfat, was prepared and designated as follows:

No. 24 – Control for 0.1% levels
No. 26 – 0.1% wheat germ oil *A*
No. 28 – 0.1% wheat germ oil *C*
No. 30 – 0.1% wheat germ oil *D*
No. 52 – Control for 0.05% levels
No. 54 – 0.05% wheat germ oil *A*
No. 56 – 0.05% wheat germ oil *C*
No. 58 – 0.05% wheat germ oil *D*

One set of samples, packed in No. 1 tins, were opened on arrival from the manufacturing plant, reconstituted, and tested for flavour and odour. All samples were perfectly fresh and it was impossible to differentiate between the various treatments. A portion of each powder was then transferred to 125 ml. Erlenmeyer flasks, stored at 65° C., and tested periodically for peroxides. The results are given in Fig. 3. All the wheat germ oils indicated definite stabilizing ability, but antioxidant *D* at a level of 0.1% of the butterfat was slightly superior to the others.

(*b*) *Storage at 65° C. after Period at Lower Temperatures*

Portions of the fresh powders were also stored in open containers at room temperature and 37° C. After several months' storage it was evident that changes other than oxidative deterioration of the butterfat had occurred; the powders, especially those at room temperature, had developed stale, musty odours and flavours, yet no increase in peroxide value great enough to account for butterfat decomposition could be observed. Even at 37° C. the samples showed a very slow increase in peroxide values, as can be seen in Table I.

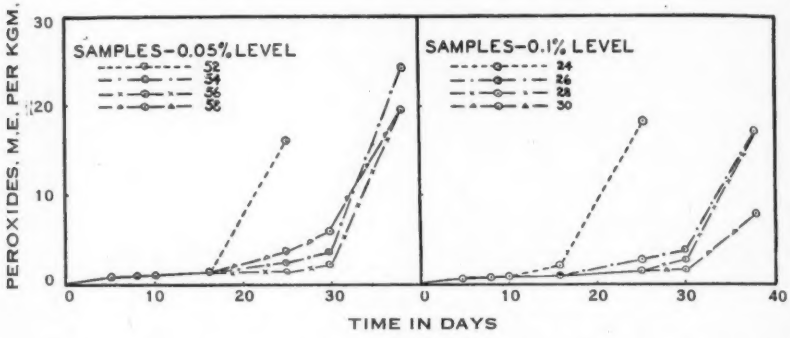

FIG. 3. *Accelerated storage test at 65° C. on fresh samples of spray-process whole milk powders.*

TABLE I

PEROXIDE VALUES OF MILK POWDERS STORED AT 37° C. EXPOSED TO THE AIR

Sample No.	Storage time, weeks						
	0	6	13	22	68	71	77
	Milliequivalents of peroxide per kgm. of milk powder						
24	1.8	2.3	2.0	1.6	0.9	2.3	3.4
26	1.1	2.5	1.1	0.5	2.5	3.6	4.1
28	1.4	2.3	1.4	0.7	5.2	10.0	12.7
30	1.3	2.5	2.5	0.7	0.5	0.9	3.1
52	0.9	0.5	2.0	0.7	0.2	1.4	0.9
54	0.8	0.7	1.4	0.5	3.2	6.1	7.0
56	0.8	0.9	2.2	0.5	0.5	5.0	7.7
58	0.6	0.5	0.9	0.6	2.2	1.1	1.1

Since these samples were not behaving in a manner similar to that of the samples in the original experiment (Fig. 3), an accelerated storage test at 65° C. was started and carried along with the experiment reported in Table I. Peroxides were determined periodically on these samples and the results, plotted in Fig. 4, show that the stabilities of the samples at 65° C. before and after storage were not related. Accelerated tests at 65° C. on the fresh powders (Fig. 3) indicated that the control samples, Nos. 24 and 52, deteriorated more rapidly than the samples containing antioxidants. However, after storage at 37° C., the results showed that samples Nos. 26, 28, 54, and 56 were the most readily oxidized. The milk powders, after storage at room temperatures, were very stable and even at 65° C. breakdown occurred only after 50 to 100 days.

To explain these anomalous results, the powders were analysed for moisture, solubility, and reducing groups. These values were compared with those for air-packed material stored for 17 months at room temperature in No. 1 tins. The results are presented in Tables II and III.

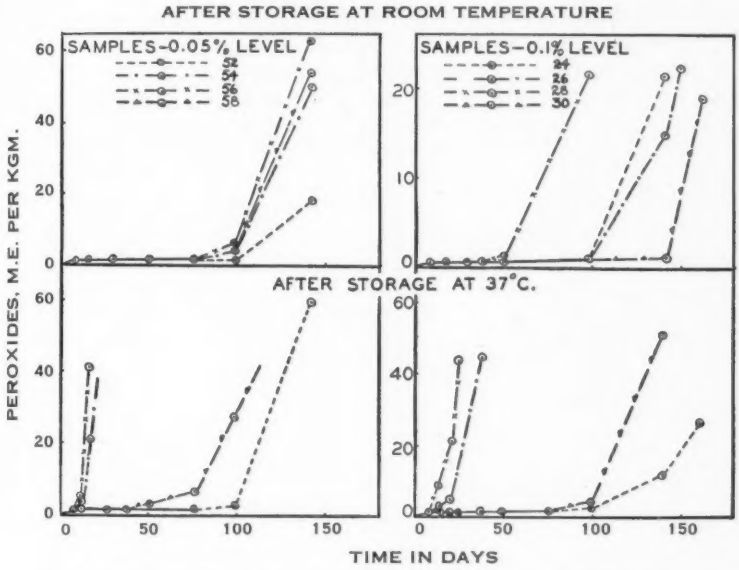

FIG. 4. *Accelerated storage test at 65° C. on spray-process whole milk powder previously stored at room temperature and 37° C.*

TABLE II

EFFECT OF STORAGE FOR 72 WEEKS ON AIR-PACKED MATERIAL IN NO. 1 TINS

Sample No.	Peroxide value	Reducing groups	Solubility, %	Moisture, %
24	4.3†	3.55*	92.5	2.20
26	2.7	3.75	92.4	3.08
28	2.5	3.75	91.9	3.74
30	2.5	3.85	92.1	3.90
52	3.8	3.55	92.3	3.74
54	2.3	3.65	92.3	3.74
56	3.0	3.20	92.9	2.54
58	4.1	4.40	92.0	2.62

† *Milliequivalents of peroxide per kilogram of milk powder.*
* *Moles* $\times 10^5$ *reducing groups per gram of milk powder.*

These results provide an explanation of the fact that the relative stability of the milk powders was now reversed. The solubility of the samples stored at room temperature had decreased more than that of the samples stored at 37° C. and the moisture content was apparently a factor. The higher values obtained in August 1943, for the moisture content of the samples at room temperature, were probably due to a higher relative humidity in the laboratory than in the 37° C. oven. The increased moisture content had more than offset the denaturing effect of the higher temperature.

TABLE III

EFFECT OF STORAGE AT ROOM TEMPERATURE AND AT 37° C.

Sample No.	Reducing groups	Solubility, %	Moisture	
			Aug. 1943	April 1944
Stored at room temperature				
24	20.3*	45.0	5.21	3.09
26	22.0	45.5	—	3.07
28	21.0	44.8	—	3.18
30	21.0	51.1	4.65	3.32
52	22.1	48.0	4.66	2.84
54	20.7	47.8	—	3.30
56	18.0	46.3	—	3.39
58	22.7	50.6	4.53	3.12
Stored at 37° C.				
24	17.5	56.2	4.23	3.08
26	14.4	70.1	—	3.23
28	14.5	68.4	—	3.44
30	19.9	48.5	4.02	2.67
52	17.8	60.1	4.10	2.96
54	15.2	81.0	—	3.40
56	15.2	82.5	—	3.35
58	17.2	67.9	3.94	3.03

* *Moles* $\times 10^5$ *reducing groups per gram of milk powder.*

The quantitative changes in the reducing groups of the milk powders were pertinent in explaining the relative stability of the samples. From an initial value of approximately 3.75×10^{-5} moles of reducing groups per gram, the powders open to the air attained values of 22.7×10^{-5} moles, with the room temperature samples showing consistently higher values. Of the samples stored at 37° C., Nos. 26, 28, 54, and 56 were lower in reducing groups than either the control or the sample with wheat germ oil *D*. They also had the shortest induction period in the accelerated test and the highest values for solubility.

Series IV—Storage Trials at 65° C. after Preliminary Storage at Room Temperature

The whole milk powders in this series were prepared with standard spray-drying equipment and were identified as follows:

Control – No antioxidant
A – 0.75% wheat germ oil *A*
AA – 1.50% wheat germ oil *A*
C – 0.75% wheat germ oil *C*
CC – 1.50% wheat germ oil *C*
D – 0.75% wheat germ oil *D*
DD – 1.50% wheat germ oil *D*

The powders were stored in fibre containers and sampled periodically for the determination of peroxides. As there was no appreciable increase in peroxide values after eight months' storage, portions were removed and subjected to an accelerated test, the results of which are shown in Fig. 5. The

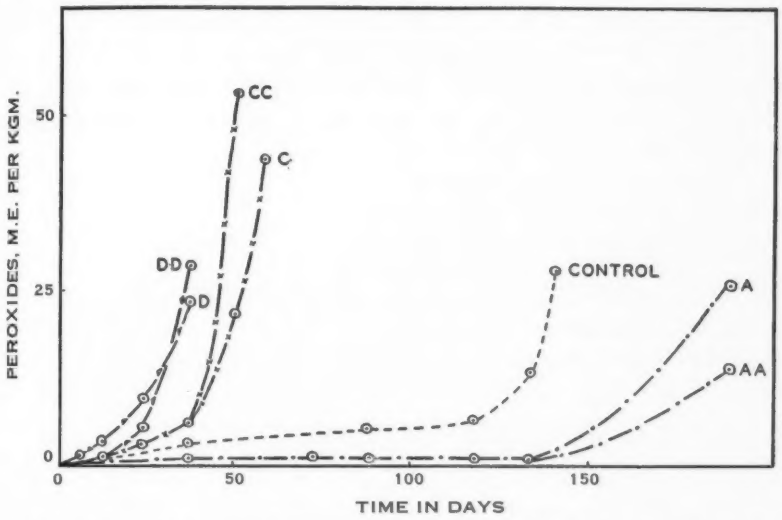

FIG. 5. *Accelerated test at 65° C. on spray-process whole milk powders previously stored at room temperature.*

samples containing antioxidant *A* were more stable than the control but the milk powders treated with antioxidants *C* and *D* deteriorated more rapidly. Reducing groups and solubilities were then determined in an attempt to ascertain the cause of this variation. The results are given in Table IV.

The samples treated with antioxidants *C* and *D* gave the lowest values for reducing groups and were the least stable. The control and powders *A* and

TABLE IV

REDUCING GROUPS AND SOLUBILITY OF POWDERS

Sample	Reducing groups	Solubility, %
Control	21.2*	57.7
A	7.1	92.6
AA	13.4	85.9
C	5.25	94.1
CC	5.30	93.8
D	3.25	93.9
DD	5.55	94.9

* *Moles* $\times$ 10^5 *reducing groups per gram of milk powder.*

AA showed a much longer induction period and also had a higher content of reducing substances. These findings were not in complete agreement with the results of similar accelerated tests reported in Series III, where the milk powders containing antioxidants *A* and *C* were the least stable. They show a disparity in the behaviour of a given antioxidant but the relation between reducing groups and stability is confirmed. Copper contamination was not appreciable, analyses of two samples showing only 1.75 and 1.81 p.p.m.

Series V—Storage Trials at 65° C.—Roller-process Whole Milk Powders

This experiment was carried out with roller-process whole milk powders containing wheat germ oil antioxidants as follows:

Control – No antioxidants
A – 0.1% wheat germ oil *B*
B – 0.1% wheat germ oil *A*
C – 0.1% wheat germ oil *C*

These samples were stored in friction-top cans at room temperature and were examined periodically. As there was no appreciable increase in peroxide values after 18 months' storage, a portion of each sample was subjected to an accelerated test at 65° C., the results of which are shown in Fig. 6.

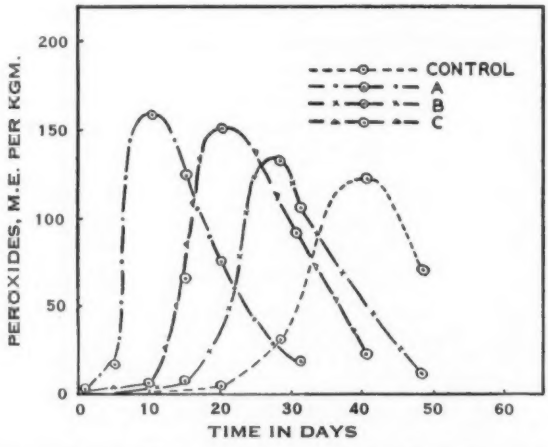

FIG. 6. *Accelerated test at 65° C. on roller-process whole milk powders previously stored at room temperature.*

The wheat germ oil actually appeared to behave as a pro-oxidant, in Samples *A*, *B*, and *C*. This experiment also showed clearly that a single peroxide determination could fail to indicate the quality of a milk powder since the peroxide values pass through a maximum. Peroxide and reducing values obtained after 27 months' storage at room temperature are given in Table V.

Sample *A* was definitely tallowy and although the others were stale they were not rancid. These roller-dried powders had a high content of reducing

substances, owing no doubt to the high temperature of processing. The presence of such antioxygenic substances probably accounts for the much longer storage life of this type of powder. The peroxide values indicated

TABLE V

REDUCING GROUPS AND PEROXIDE VALUES AFTER 27 MONTHS' STORAGE

Sample	Reducing groups	Peroxide values
Control	27.4*	0.7†
A	25.9	23.6
B	27.7	0.9
C	25.9	0.9

* *Moles* $\times$ 10^5 *reducing groups per gram of milk powder.*
† *Milliequivalents per kilogram of milk powder.*

that the samples were deteriorating in the same order as shown in the accelerated test. The average copper content of these powders was 3.75 p.p.m.

THE EFFECT OF HEAT AND MOISTURE ON THE DEVELOPMENT OF REDUCING GROUPS

It is known that the solubility of milk powders with a high moisture content rapidly decreases during storage and that this effect is accompanied by the development of stale, musty odours and flavours. An experiment was therefore carried out to ascertain the effect of high moisture content on reducing groups and peroxide values.

Milk powders were held at 75% relative humidity by placing them in a large desiccator containing glycerol solution of sp. gr. 1.121 (10). Reducing groups, peroxide values and solubilities were determined at intervals, with the results shown in Tables VI and VII. The effect of storage under these conditions was to decrease the solubility and peroxide value and increase the content of reducing substances.

TABLE VI

EFFECT OF STORAGE IN A HUMID ATMOSPHERE ON THE REDUCING GROUPS

Sample No.	0 days	11 days	20 days	36 days
24	3.55*	4.95	8.45	11.80
26	3.75	5.25	8.15	11.55
28	3.75	4.95	9.10	12.50
30	3.85	5.10	8.25	11.80
61	6.35	6.50	10.35	11.95
63	6.60	7.40	10.20	11.75

* *Moles* $\times$ 10^5 *reducing groups per gram of milk powder.*

TABLE VII

THE EFFECT OF STORAGE UNDER HUMID CONDITIONS ON MOISTURE, PEROXIDE VALUE, AND SOLUBILITY

Sample No.	Solubility, %		Peroxide		Moisture, %	
	Original value	After storage	Original value	After storage	Original value	After storage
24	92.5[1]	44.9	4.3†	0.7	2.20	9.02
26	92.4	45.1	2.7	0.4	3.80	8.95
28	91.9	45.3	2.5	0.7	3.74	9.29
30	92.1	44.7	2.5	0.2	3.90	7.54
61	70.0	46.6	1.3	0.2	3.06	8.61
63	67.5	45.1	1.0	0.4	3.05	9.47

[1] *These values have been reported previously but are included here for comparison.*
† *Milliequivalents of peroxide per kilogram of milk powder.*

Evidently the higher concentration of reducing substances was responsible for the decrease in peroxide values. These samples finally developed stale flavours and a very definite "cheesey" aroma, which was probably due to micro-organisms. The effect of heat was ascertained by determining reducing groups in these same samples during an accelerated test at 65° C. The results in Table VIII show that there was a relatively rapid increase in reducing groups during the initial period of storage, followed by a more gradual increase.

TABLE VIII

THE EFFECT OF STORAGE AT 65° C. ON THE REDUCING GROUPS

Sample No.	Reducing groups[1]		
	12 days	21 days	33 days
24	11.6*	13.7	13.5
26	12.2	14.3	13.9
28	10.5	13.5	13.6
30	13.1	13.5	14.2
61	14.0	17.5	19.1
63	14.3	16.9	18.7

[1] *Original values of these powders are given in Table VI.*
* *Moles* $\times$ 10^5 *reducing groups per gram of milk powder.*

THE EFFECT OF REDUCING GROUPS ON THE RIBOFLAVIN CONTENT

Since riboflavin is readily destroyed by reduction, it seemed probable that the presence of reducing groups might have some effect on the vitamin B_2 content of the milk powder. A number of analyses were therefore made on representative samples by the method of Hand (4) adapted for use with milk powder.

The only apparent effect of a high concentration of reducing substances was noted in Series III. The samples that had been stored open to the air, at room temperature, had an average riboflavin content of 11.6 μgm. per gm. The milk powders stored at 37° C. and at room temperature in sealed containers averaged 13.2 and 13.3 μgm. per gm., respectively. The results shown in Tables II and III indicate that the concentration of the reducing groups was much higher in the samples in which there was a definite decrease in riboflavin content. It therefore appeared probable that these reducing substances were responsible for the destruction of the vitamin.

Discussion and Conclusions

Accelerated tests at 65° C. give an accurate indication of the relative keeping qualities of whole milk powders. Only chemical tests can be used under such conditions because caramel-like flavours interfere with organoleptic examination. Samples of wheat germ oil had a considerable peroxide content but the removal of the peroxides did not improve the antioxygenic properties of the oil. Wheat germ oil alone and in combination with citric acid and lecithin was effective in inhibiting oxidation catalyzed by copper contamination. In powders of normal copper content, the increase in reducing substances that occurred during prolonged storage open to the air offset the effect of the antioxidants.

There was a rapid increase in reducing substances in milk powders stored in a humid atmosphere or at elevated temperatures. The relative stability of samples in a particular series was related to their content of reducing substances. The concentration was found to be much higher in roller-dried powders than in spray-dried samples, probably owing to the higher temperatures employed in the former process. This may be responsible for the greater stability of roller-dried products. The peroxide content was of value in following the progress of oxidative deterioration but it was also necessary to determine the concentration of reducing groups since these were likewise associated with off-flavours.

Harland and Ashworth (5) have recently used thiamin disulphide for the estimation of reducing substances in processed milk. They state that certain reducing compounds are liberated from liquid whole-milk and skim-milk powder at temperatures above 70° C. but if the heating were continued for 10 min. at 80° C., or at higher temperatures, the concentration decreased. These findings are contrary to the results obtained in this laboratory by the ferricyanide method (2). We have found that reducing substances are present in raw milk, and the amount increases on heating at temperatures up to 100° C. In addition, heated skim-milk powder and roller-process whole milk powder contained a much higher concentration of reducing substances than the original milk. Evidently the reagents are estimating different types of reducing compounds. In this investigation the storage tests were continued beyond the stage at which the samples were palatable because it appeared of

value to determine the factors that were responsible for the chemical changes in the advanced stages of deterioration.

Acknowledgments

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DRIED MILK POWDER

IV. THE EFFECT OF STORAGE TEMPERATURE, MOISTURE CONTENT, AND PLANT SOURCE ON THE KEEPING QUALITY OF MILK POWDERS OF DIFFERENT FAT LEVELS[1]

By W. A. Bryce[2] and J. A. Pearce[2]

Abstract

Milk powders with fat contents of 1, 26, 28, and 30% from two plants were tempered to moisture contents of 2, 3, and 5% and stored for periods up to 16 weeks at temperatures of from 40° to 140° F. Appreciable deterioration, assessed by palatability, occurred in the whole milk powders stored at temperatures of 60° F. and higher, and there was considerable difference in the stability of powders from the two plants. For both plants, the keeping quality of powders of 26 and 28% of butter fat was equal. At 80° F. and lower, the powder containing 30% of butter fat was more stable than the 26 and 28% powders from the same plant, but at higher temperatures the 30% powder deteriorated more rapidly. At 80° F. the average decrease in palatability of whole milk powders with 2% moisture was two palatability units. The palatability of the skim-milk powder increased greatly at all temperatures during the early part of the storage period, but later decreased at temperatures of from 100° to 140° F. Skim-milk powder of 2% moisture stored at 80° F. had a palatability score 2.5 units higher than the initial score. In general, a moisture content of 3% was preferable to moisture contents of 2 and 5% for both whole and skim-milk powders. The differences in stability of powders from different plants were enhanced by increased moisture contents and higher storage temperatures.

Introduction

Experience gained during the war years has emphasized that improved methods of handling and storing must be developed if dehydrated foods are to assume their proper place in the post-war period. The importance of dried milk in the national diet has focused considerable attention on studies of production methods and keeping quality of this material.

Studies on the effect of temperature on the development of storage flavours in dried milk have yielded conclusions that have been somewhat conflicting. In one investigation (1) little difference was observed between powders stored at 40° F. and at 68° F., but a marked difference at 100° F. was reported. Powders from partially skimmed milk did not develop tallowiness when stored for 18 months at 32° F., but at 68° F. this off-flavour was noticeable in from five to six months. The rate of deterioration of whole milk powders has been found to increase rapidly at temperatures above 32° F. (4). It has also been reported (9) that the palatability decreased more rapidly at 117° F. than at 100° F. A storage temperature of 100° F. has been found to be better than either 80° or 120° F. (7).

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[2] *Biochemist, Food Investigations.*

The effect of moisture on the keeping quality of both spray and drum-dried powders has been studied by a number of investigators (1, 2, 4, 9) who have found that moisture contents below 2% accelerated the development of talliness in spray-dried powders, and that at moisture contents above 5%, deterioration occurred rapidly in powders of all types. Little difference was observed in the effect of moisture contents between 2.3 and 5.4% on the development of tallowy flavours in powders of 30% butter fat, but with powders of 28% fat, a moisture content of 3.5% was preferable to either 2 or 5%. In general it has been found that the optimum moisture content required to reduce the development of storage flavours to a minimum is between 2 and 3%. It has been recognized that the keeping quality of dried milk is a function of both storage temperature and moisture content, increased moisture contents accelerating the effect of high storage temperatures.

It is evident that some disagreement exists in the published data on the keeping quality of milk powders. This paper discusses an investigation designed to evaluate the effects of moisture content and storage temperature on keeping quality, and at the same time to consider the effects introduced by different processors, and by the use of powders of different fat levels.

Materials and Methods

The powders used were commercial spray-dried products made by two Canadian companies from milk produced in the spring of the year. Powders of 1, 26, and 28% of fat were supplied by one plant, and of 1, 26, 28, and 30% of fat by another.

Each type of milk powder was divided into three portions, one of which was tempered to a moisture content of 2% by vacuum desiccation over phosphorus pentoxide, and was stored in tin plate (air as headspace gas) at temperatures of 40°, 60°, 80°, 100°, 120°, and 140° F. (4.4°, 15.6°, 26.8°, 37.8°, 49°, and 60° C.). The moisture contents of other portions were adjusted to 3 and 5%, respectively, by exposure to an atmosphere of high humidity, and were stored in a manner similar to those at 2% moisture, but at temperatures of 80°, 100°, and 120° F. only.

Palatability was the only quality test used that was satisfactory (7). The powders were tested for quality both initially and after storage for 2, 4, 8, and 16 weeks by an organoleptic method previously reported (7). The reconstituted milk was scored by a panel of 14 tasters and given a rating of from 10 to zero, 10 being the equivalent of best quality fresh whole or skim-milk.

Results

Effect of Temperature

The palatability results were analysed by statistical methods. The significant effects of different temperatures on each of the whole milk powders of 2% moisture are shown graphically in Fig. 1, and a summary showing averages for all products stored at the different temperatures is presented in

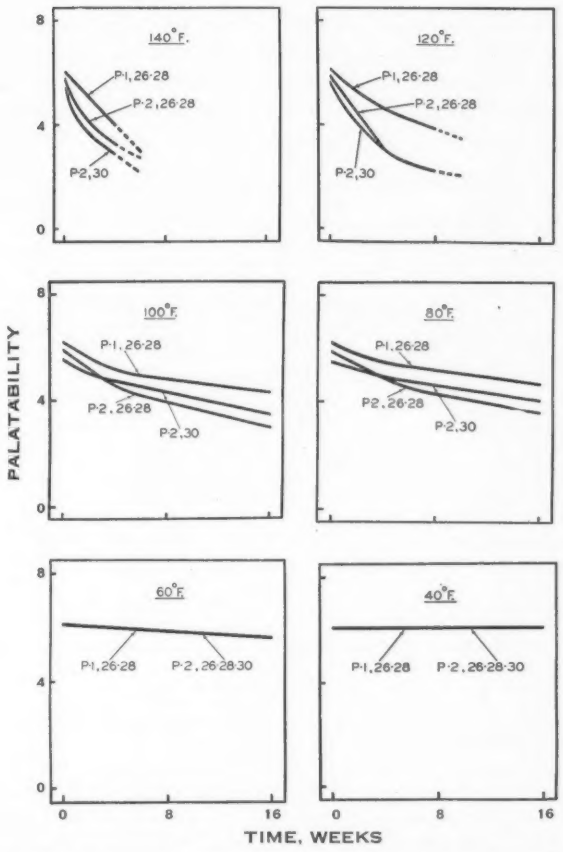

FIG. 1. *The effect of storage temperature on the palatability of whole milk powders of different fat levels from Plants 1 and 2. All samples at 2% moisture.*

Fig. 2. Although there was an appreciable difference between powders from the two plants, the powders of 26 and 28% butter fat from either of the plants behaved in a comparable manner at each storage temperature.

At the higher temperatures of 80° to 140° F., the material from Plant 1 was given a consistently higher palatability score than that of comparable fat content from Plant 2, showing that the product from Plant 2 was less stable than that from Plant 1. At the lower temperatures, the behaviour of all powders was the same. At 80° and 100° F., the 30% butter fat powder was given a higher palatability rating than the 26 and 28% powders from the same plant. At 120° and 140° F. the 30% powder was scored lower than the 26 and 28% powders.

Storage for 16 weeks at temperatures of 60° F. and lower had little effect on the palatability of all whole milk powders investigated, although a slight

downward trend was observed at 60° F. Typical storage flavours developed in powders at temperatures of 80° F. and higher, the rate of deterioration increasing with temperature. The data in Fig. 1 do not agree with observations previously reported (6, 7, 10) that milk powder maintained a higher palatability when stored at 100° F. than at either 80° or 120° F.

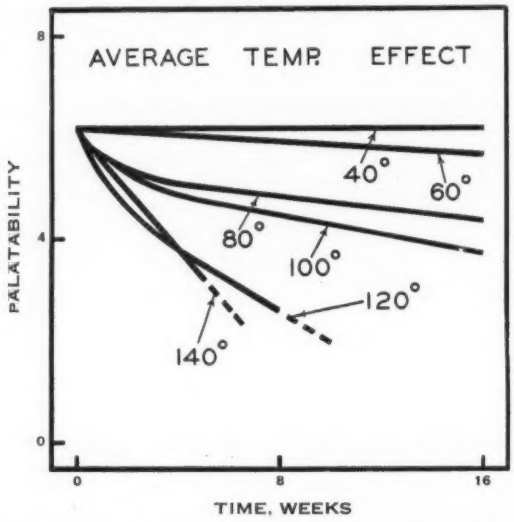

FIG. 2. *The effect of storage temperature on the palatability of whole milk powders (averages of all samples) at 2% moisture.*

The existence of an induction period in the deterioration of milk fat has been reported (5), but the present data gave no indication of such a phenomenon when palatability was used as a quality test. It can be seen from Fig. 1 that the rate of deterioration of all whole milk powders at all temperatures approximated a straight line relation, although the decrease in the rate of deterioration previously observed (7, 10) was noticeable. If an induction period existed, it must have come within the first two weeks of storage.

A comparison of the deterioration in skim-milk powder from the two plants is shown graphically in Fig. 3, and a summary of the temperature effects is presented in Fig. 4. Considerable difference was observed in both the initial palatability and the changes occurring during storage. The data for 40° and 60° F. have been combined as there was no real difference between the values for the powders at these two temperatures.

In contrast to the results obtained from the study of whole milk powders, it was found that the skim-milk powder from Plant 2 was much superior in keeping quality to that from Plant 1. However, throughout the experiment, the over-all behaviour of the two skim-milk powders was almost parallel at all temperatures. Under all conditions both products exhibited an initial

increase in palatability over the first four weeks of storage. At temperatures of 140°, 120°, and 100° F. the trend was downward after this initial increase was past, but below 100° F. there was no appreciable change during the next 12 weeks. The average palatability of both powders rose to a higher value as the temperature decreased (Fig. 4).

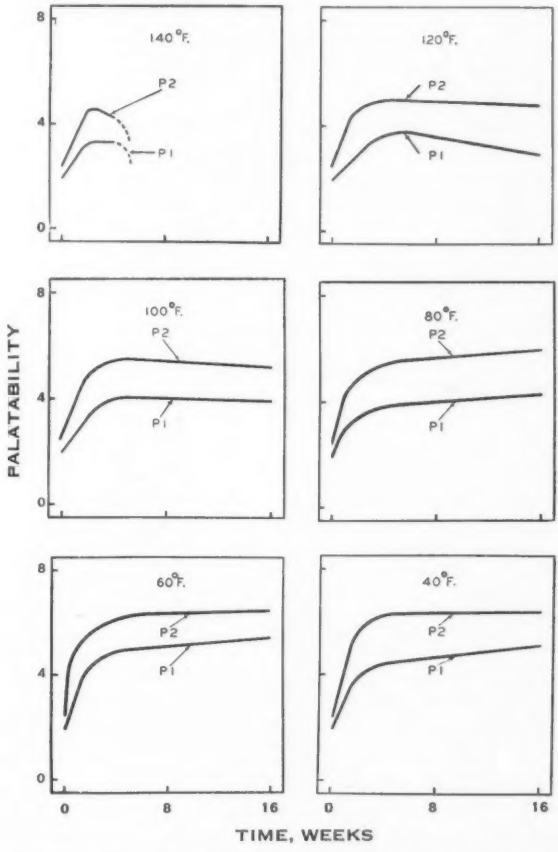

FIG. 3. *The effect of storage temperature on the palatability of skim-milk powders from Plants 1 and 2. All samples at 2% moisture.*

Effect of Moisture

The significant effects of moisture content on the keeping quality of dried whole milk powders are shown in Fig. 5. As in Fig. 1, the data for powders of 26 and 28% butter fat are combined, as the behaviour of these powders was identical. The different behaviour of the 30% butter fat powder was observed at all moisture levels. At 80° F. the 30% powder was given a

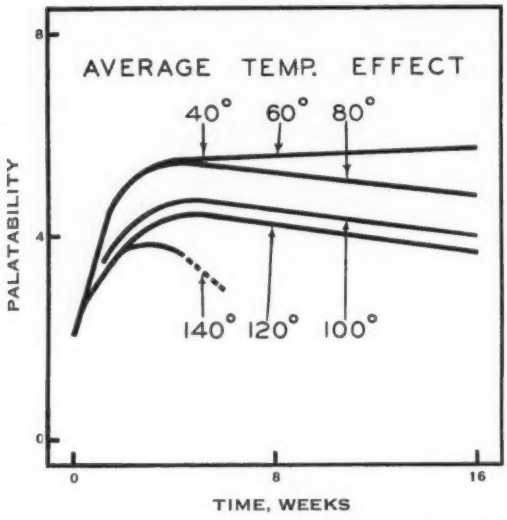

FIG. 4. *The effect of storage temperature on the palatability of skim-milk powders (averages of all samples) at 2% moisture.*

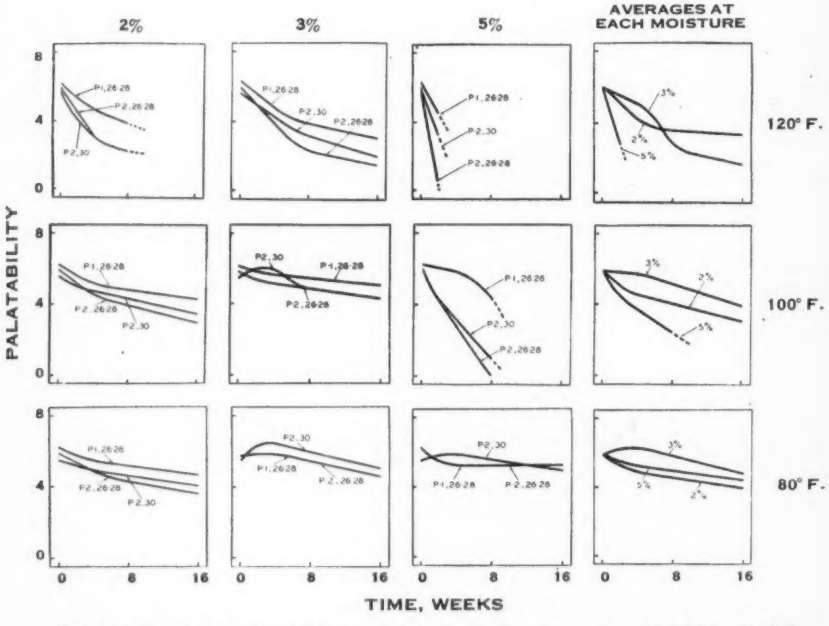

FIG. 5. *The effect of moisture content and storage temperature on the palatability of whole milk powders from Plants 1 and 2.*

slightly higher palatability rating than the 26 and 28% fat powders from the same plant. For all moisture levels, at higher temperatures, the difference in origin of the powders introduced greater variations than did the difference in fat content. The variation in stability of the powder with plant source was enhanced by increased moisture contents and by higher storage temperatures. A comparison of powders of equal fat levels at 100° and 120° F. showed that the keeping quality of powder from Plant 1 was superior to that of the powder from Plant 2 at all moisture levels.

The effect of moisture on the keeping quality of the powders was marked. At 120° F. the palatability of the powders of 2 and 3% moisture decreased at comparable rates, but those at 5% moisture deteriorated very rapidly. Considerable browning of these latter powders was observed after only two weeks of storage, and the palatability had decreased to such an extent that no further tasting was done. At 100° F. the powders with 3% moisture were markedly superior to those with moisture contents of 2 and 5%. At 80° F. the quality of the 3% moisture powders was appreciably better than that of the 2 and 5% powders although the differences were not as great as they were at 100° and 120° F. Only at 80° F. did the quality of the powders of 5% moisture remain higher than that of the powders of 2% moisture.

The combined effect of moisture content and storage temperature on keeping quality of whole milk powder can readily be seen from the graphs in Fig. 5, which show averages for all powders at each moisture level. At temperatures of 80° and 100° F., the superiority of the 3% moisture powder was evident. This was also evident during the first eight weeks of storage at 120°, but by the end of the sixteenth week the palatability of the 3% moisture powder had dropped below that of the 2% powder. Both the rate of deterioration shown by the slope of the curve and the actual palatability level of the powder were dependent on moisture content and storage temperature.

The data for the study of moisture effects on the keeping quality of skim-milk powders are shown graphically in Fig. 6. The superiority of the powder from Plant 2 over that from Plant 1 was observed for all moisture levels. The palatability change in the two products was about the same, the principal difference between them being in the initial quality.

As shown by the graphs of average palatabilities at each moisture level, the powders at a moisture content of 3% were again found to be superior to those at 2 and 5%, although the difference between the 2 and 3% powders was not marked. Storage life of the powder containing 5% moisture was considerably shortened at 120° and 100° F. but at 80° F. this powder was scored as high as were the 2 and 3% powders.

The marked initial increase in palatability exhibited by the skim-milk powder with a 2% moisture content was also observed for the 3 and 5% powders. At 80° F., this increase continued in powders of 2 and 5% moisture throughout the storage period. The level to which the palatability of the 5% powders rose was found to decrease with increasing temperatures.

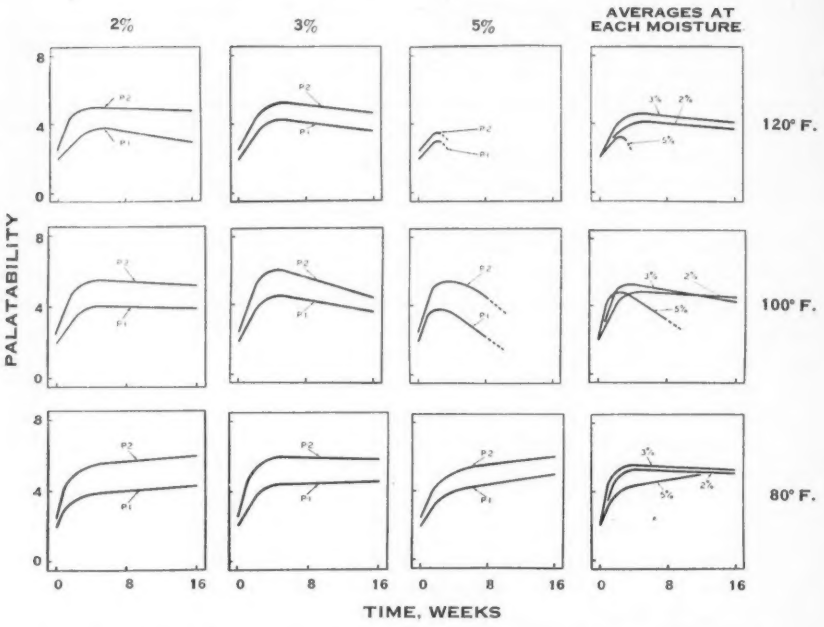

FIG. 6. *The effect of moisture content and storage temperature on the palatability of skim-milk powders from Plants 1 and 2.*

Discussion

This work has shown that considerable difference existed between plants in the effect of temperature on the keeping quality of whole milk powders. Deterioration was measurable at temperatures of 60° F., the rate of deterioration increasing with temperature and approximating a straight line relation. For skim-milk powders the marked difference in initial palatability between plants was uniformly maintained throughout the storage period. For both plants at all temperatures the palatability of skim-milk powders increased during the early part of the storage period.

Both whole and skim-milk powders at a moisture content of 3% maintained a higher palatability under storage than did these powders at moisture contents of 2 or 5%. Increased moisture contents were found to enhance the difference existing in the stability of powders from different plants.

The increase in palatability with storage of skim-milk powders has not been satisfactorily explained. It may be the result of the recombination in the powders of materials formed during early processing that was responsible for the low initial palatability. Protein degradation products such as various amines and dipeptides may recombine to form polypeptides whose flavours are not objectionable. At 80° F. this recombination may be sufficient to off-set the normal deterioration due to storage, and hence at this temperature

the palatability continues to increase throughout the entire storage period. Another explanation is that the concentration of these undesirable products may have been reduced by volatilization during storage (8). Further study of this problem is being made at the present time.

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THE KEEPING QUALITY OF DEHYDRATED MIXTURES OF EGG AND MILK[1]

By Jesse A. Pearce[2], Joan Whittaker[3], H. Tessier[4], and W. A. Bryce[2]

Abstract

The storage life of a dehydrated mixture of egg and milk, when assessed by both palatability and fluorescence measurements, was shorter than the life of milk powder of similar protein, fat, and carbohydrate content. Increased quantities of egg in the mixture decreased the quality of the mixture, both initially and during 16 weeks' storage. These effects were noticeable at all temperatures studied between 40° and 140° F. but were most marked above 80° F. After 16 weeks at 80° F., material packed under carbon dioxide usually had better palatability than the air-packed products. The effect of added sugar was most noticeable at 120° and 140° F. Lactose had a slightly beneficial effect; sucrose was more effective.

Introduction

Preliminary data obtained in these laboratories indicated that dehydrated mixtures of milk and egg were less stable during storage than were either of these components separately. This becomes a matter of importance if these dehydrated mixtures are to be prepared for satisfactory reconstitution into ice cream, custard mixes, milk shakes, or other similar foods. Mixes such as these are commercially available in Canada and are also being used to prepare ice cream for the American armed services (12). Possible use by the Canadian armed forces led to a study of their keeping quality.

The addition of sugar to milk before dehydration was believed to improve the keeping quality of the product (4) and the addition of sucrose to eggs before drying retarded fluorescence development (11), which in turn is related to egg powder quality (9). Therefore, the preservative effect of sucrose and lactose on these materials was evaluated, since products of the type described are usually used in the preparation of sweetened dishes and the addition of a small amount of sugar to the liquid before drying would not affect its use.

Packing milk powder under an atmosphere of carbon dioxide was reported to extend the storage life of this product (5), although recent evidence indicated that any beneficial effect was not evident to a taste panel (14). Egg powder when packed under this gas deteriorated less rapidly than air-packed material (8). It was desirable in this study to obtain additional information about the effect of gas-packing by comparing carbon-dioxide–packed with air-packed mixtures.

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Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as paper No. 152 of the Canadian Committee on Food Preservation and as N.R.C. No. 1363.

[2] *Biochemist, Food Investigations.*

[3] *Captain, R.C.A.M.C.*

[4] *Laboratory Steward, Food Investigations.*

Materials and Methods

Mixtures Used and Storage Conditions

The materials used were obtained from commercial Canadian sources. Some formulas were prepared by mixing, and dehydrating, using a laboratory spray drier (15); others, by mixing the ingredients in the dry state. Differences in moisture content of dried egg powder (13) and dried milk powder (3) affect the keeping quality of both of these products. Therefore, the moisture content of the mixtures was kept within a range usually satisfactory for both dehydrated egg and milk (2 to 4%).

TABLE I

CALCULATED FAT, PROTEIN, AND CARBOHYDRATE PERCENTAGES OF VARIOUS SPRAY-DRIED EGG AND MILK MIXTURES

(Products at 2.5% moisture)

Mixture	Fat	Protein	Carbohydrate	Ash
(*A*) 2.4 lb. of skim-milk powder in 20 lb. of fresh whole milk	15	31	47	4.8
(*B*) 1 doz. fresh eggs, 2.4 lb. of skim-milk powder, in 20 lb. of fresh whole milk	20	35	38	4.4
(*C*) 5 doz. fresh eggs, 2.4 lb. of skim-milk powder in 20 lb. of fresh whole milk	30	42	22	4.0
(*D*) 1 lb. of lactose, 5 lb. of fresh eggs, 1 lb. of skim-milk powder in 15 lb. of fresh whole milk	21	30	41	4.5
(*E*) Dry mix of whole milk powder, whole egg powder, dried fermented egg albumen, and lactose in proportions of 147: 78: 76: 125	17	33	45	3.5
(*F*) Dry mix of whole milk powders, skim-powder and whole egg powder in proportions of 147: 200: 78	17	35	39	6.4

The formulas investigated and the calculated analyses are given in Table I. Formula *A* gave a mixture without egg; *B* gave a mixture with a moderate amount of egg; and *C* showed the effect of adding a large amount of egg; while *D* gave a mixture with a high egg level, but of a composition similar to *B*. Formula *F* provided a dry-mixture approximating *B*, and *E* was a similar dry-mix with skim-milk protein replaced by egg protein. The effect of sugar on the keeping properties of Formulas *A*, *B*, and *C* was evaluated by drying these mixes after the addition of 3% of lactose or 3% of sucrose. While these were only a few of the possible combinations they were representative of the products likely to be used.

All mixtures were packed in hermetically sealed tin plate containers with air in the headspace and stored at 40°, 60°, 80°, 100°, 120°, and 140° F. (4°, 16°, 27°, 38°, 49°, and 60° C.). Some were packed in tin plate under carbon dioxide and stored at 80° F.

Analytical Methods

The quality of products such as these is dependent primarily on their acceptability when reconstituted as milk shakes, custards, ice cream, or other similar dishes. Therefore, the average palatability score, determined by a panel of 14 tasters, was used for assessing quality. The material was reconstituted as a milk shake mix in a manner similar to that described for milk powder (7), but using one part solids and three parts of water by weight. The products when submitted to the tasters were sweetened but unflavoured, and to prevent differences in sweetness affecting tasters' judgment, all samples were made up to a level of 7% sweetness in terms of sucrose, utilizing recently tabulated sweetness relations (2).

The scoring technique differed from that used in previous work (7, 9). A freshly prepared mix of Formula *F* was used as the standard for tasting purposes and given an arbitrary score of 10. A score of 20 indicated a product twice as acceptable as this formula, a score of 5 indicated the product half as acceptable, and a score of 0 indicated an unacceptable mix. A fresh standard sample was used as a reference whenever palatability was determined.

An attempt was made to follow solubility changes in the mixtures by a standard procedure (1). However, during storage, the decrease in solubility of all products was so slight that the test was discontinued.

The fluorescence of a saline extract of defatted egg powder has been related to the palability of this product (9), and has also shown some relation to milk powder quality (6). Later work showed only a slight correlation between this test and the palatability of milk powder (7). Nevertheless, a modification of this test (10) was applied to some of the present samples. Correlation of fluorescence values of the stored mixes with the corresponding palatability scores was lower than that obtained for egg powder alone (9). This test was discontinued since the correlation was only .4 for the most desirable sample, Formula *A*. However, the fluorescence data were of some interest and are recorded here.

The data were subjected to analyses of variance and the significant effects were selected for discussion.

Results

Palatability Changes During Storage

The palatability changes in the basic mixtures during 16 weeks' storage at temperatures from 40° to 140° F. are shown in Fig. 1. Throughout the experiment the product prepared entirely from milk solids was considered superior, both initially and after storage. At 40° and 60° F., the mixture prepared entirely from milk and the dry mix of egg powder and whole and skim-milk powder deteriorated about equally, but less rapidly than other mixtures containing egg. At the higher temperatures (80 to 140° F.) all mixtures containing egg spoiled more rapidly than the mixture prepared only from milk. At temperatures of 100° to 140° F. the material containing the greatest quantity of egg became inedible more rapidly than those containing

little or no egg. The addition of egg to milk before dehydration reduced the initial quality of the dried product and its subsequent storage life. However, the dehydrated mixture appeared to have a better storage life than has been observed for egg powder (8).

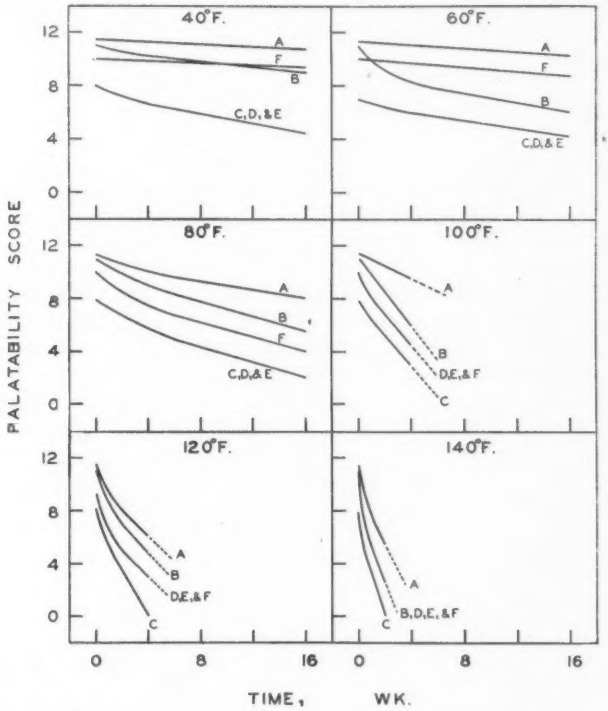

FIG. 1. *Effect of storage temperature on the palatability of stored dehydrated mixtures of egg and milk.*

The average values for samples with and without added sugars are shown in Fig. 2. Generally, there was a slight protective action as a result of the addition of sugar. The behaviour was not regular at all temperatures nor for all mixes (notably Mixture *B* at the higher temperatures), nevertheless, added sucrose was more effective than added lactose.

Formula *D* was similar to Formula *C* since it gave a dried product with a large quantity of egg solids, and was similar to Formula *B* in that it yielded a product with almost the same fat, protein, and carbohydrate ratio. This mixture had slightly better keeping quality at the higher temperatures than Formula *C* (Fig. 1). It was believed that this improvement was the result of adding lactose rather than the result of balancing the component ratio.

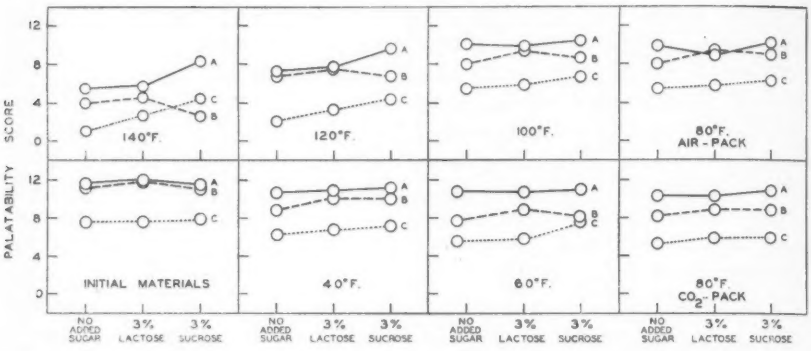

FIG. 2. *Average effect of added lactose and sucrose on the palatability of stored dehydrated mixtures of egg and milk.*

The comparative effects of packing some of the mixtures under atmospheres of air and carbon dioxide are shown in Fig. 3. It was observed that carbon dioxide provided some protection to the products *A*, *B*, and *D*, i.e., protection was afforded to mixtures of about the same composition even if eggs were

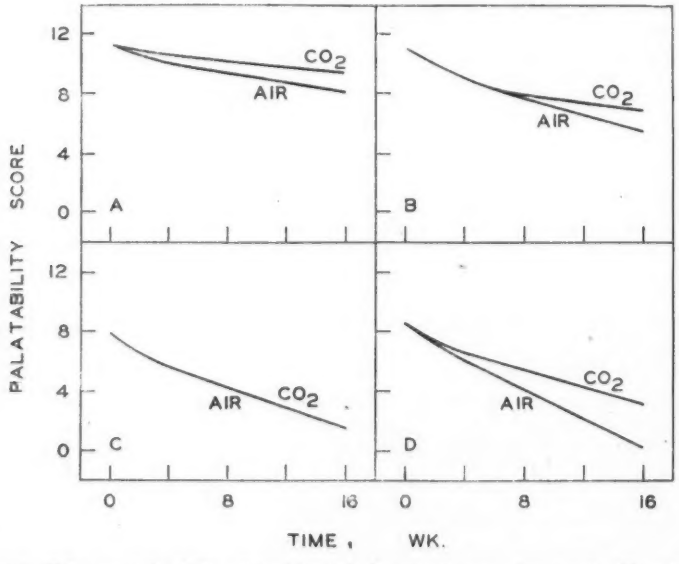

FIG. 3. *Effect on palatability of packing dehydrated mixtures of egg and milk under an atmosphere of carbon dioxide; storage at 80° F.*

present, but the life of material with high fat and protein from egg sources was not extended by carbon dioxide. The beneficial action of carbon dioxide was not pronounced; after 16 weeks' storage at 80° F. the palatability of

three of the gas-packed materials was about two units higher than that of the air-packed material.

Fluorescence Changes During Storage

As previously stated, fluorescence changes were measured during the initial portion of the experiment. In general, fluorescence increased with storage time and with temperature (Fig. 4). The initial fluorescence value was lower

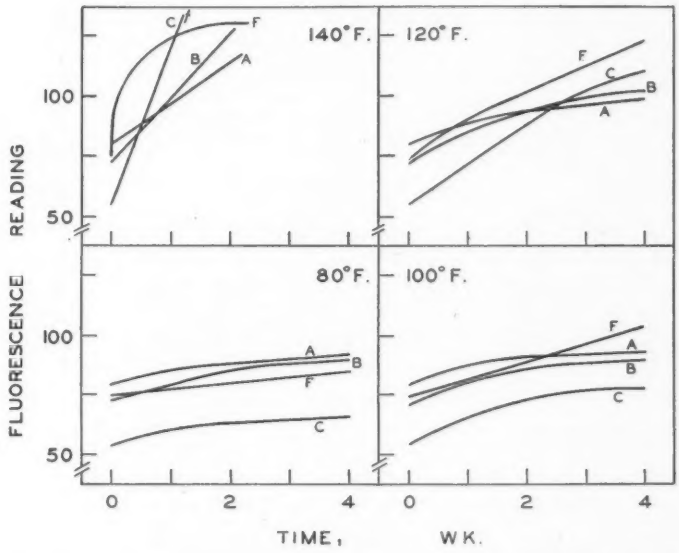

FIG. 4. *Effect of storage temperature on the fluorescence reading of saline extract of stored dehydrated mixtures of egg and milk.*

for mixes of higher egg content but the fluorescence value of these samples increased rapidly. Although parallel behaviour was usually noted for fluorescence increases and palatability decreases, the correlation between the two measures was less than that for egg powder alone (9).

One point of interest arises from comparison of Formulas *B* and *F*. At 100° to 140° F. the fluorescence of the dry-mix increased much more rapidly than that of the product prepared from the wet-mix, although the palatability measurements indicated about equal deterioration in these two materials.

Sucrose addition caused slight reduction in the average fluorescence value of the samples stored at temperatures of 80°, 100°, 120°, and 140° F. (Fig. 5). The greatest effect was apparent at 140° F.; sucrose caused the most pronounced reduction in fluorescence in the sample containing the greatest quantity of egg. This corresponds fairly well with similar observation from palatability scores (Fig. 2). The fluorescence measurement also indicated that sucrose was more effective than lactose.

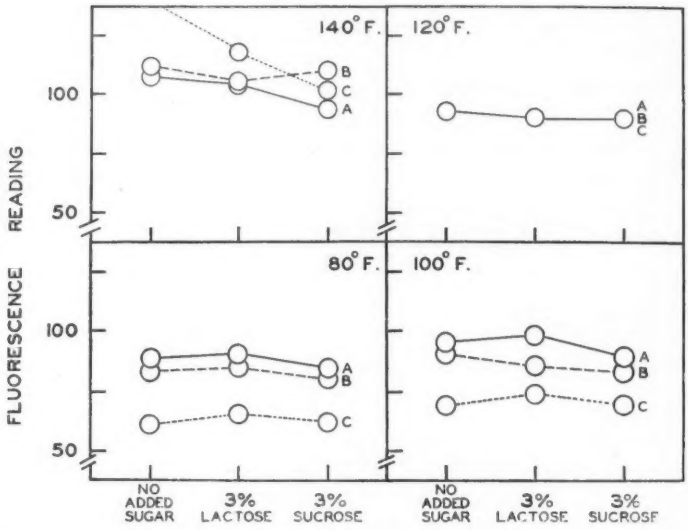

FIG. 5. *Average effect of added lactose and sucrose on the fluorescence reading of saline extracts of stored dehydrated mixtures of egg and milk.*

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THE OCCURRENCE AND DISTRIBUTION OF *SALMONELLA* TYPES IN FOWL

I. ISOLATION FROM HENS' EGGS[1]

F. E. CHASE[2] AND M. L. WRIGHT[2]

Abstract

Using selective media, an examination of 2400 hens' eggs was made to determine the incidence of *Salmonella* types in the egg or on the shell. The contents of 1000 eggs laids by a flock of presumably normal hens did not yield any representatives of the *Salmonella* group. Of a similar number of eggs produced by a flock of 55 known pullorum reactors, 61 were found to contain *S. pullorum;* no other *Salmonella* organisms were isolated. The exteriors of an additional 400 eggs obtained from the latter source failed to produce any *Salmonella* types.

Introduction

War-time conditions have brought about a tremendous expansion in the production and use of dried whole egg powder. The reported occurrence of a number of *Salmonella* types in the commercial product (5), although apparently not constituting a dangerous hazard to health (6), has served to direct interest towards the discovery of the source of these contaminants, since their presence in food products is certainly most undesirable. Gibbons and Moore (5) have presented circumstantial evidence to indicate that hens' eggs may be the source, not only of *S. pullorum*, but of other *Salmonella* types occurring in egg powder.

There is considerable evidence to implicate other types of fowl. Tanner (15) reviewed briefly a number of reports of *Salmonella* food poisoning in which ducks' eggs were thought responsible, and Scott (14) found three flocks of ducks in which one or more of the birds laid eggs containing *Bacillus aertryke.* Edwards (3) cited the report of a food poisoning outbreak affecting 20 persons in which pigeons' eggs infected with *S. typhi-murium* var. *copenhagen* were involved. Hinshaw (9) reported the presence of *S. typhi-murium* in the ovaries, oviduct, and eggs of turkeys. For the most part, however, hens' eggs have escaped suspicion, and no reference has been found that describes the isolation of any *Salmonella* type other than *S. pullorum* from hens' ovaries or the contents of their eggs.

Because of the number of types of *Salmonella* reported in dried whole egg powder, and in the visceral organs and fecal matter of chickens, and occasionally in hens suffering from enteric infections (3, 10, 13), it was decided to determine to what extent these types were present either within the egg or in fecal contamination on the shell. This paper deals with an examination of 2400 hens' eggs for the presence of *Salmonella* types.

[1] *Manuscript received August 2, 1945.*

Contribution from the Department of Bacteriology, Ontario Agricultural College, Guelph, Ont., with financial assistance from the National Research Council of Canada.

[2] *Assistant Professor.*

Methods

Because a number of mixed *Salmonella* infections in fowls have been reported (2) it was decided to include in this study 1000 eggs produced by a flock of 55 known pullorum reactors. Another 1000 eggs were obtained from a source where every effort is made to maintain normal birds. For the sake of brevity these eggs will be referred to as "diseased" and "normal," respectively, to indicate their source. The required number of fresh eggs were obtained at weekly intervals and held at room temperature until cultured.

The procedure used in culturing the eggs was based on recommendations made by Gibbons*. The eggs were soaked for approximately five minutes in a "Diversol" solution, containing 500 p.p.m. of available chlorine, after which they were rinsed in alcohol and placed on a sterile metal rack until dry. The ends were painted with double strength tincture of iodine and the eggs were again allowed to dry. Using aseptic precautions an egg was picked up, each end opened with a flamed awl, and then placed on the mouth of a 100 ml. sterile Erlenmeyer flask containing broken glass. Compressed air filtered through sterile glass wool was used to blow the contents into the flask. After thorough mixing, the egg meat was transferred to the culture medium.

As this investigation was concerned only with members of the *Salmonella* group, selective media were utilized when culturing the eggs in order to reduce possible interfering types. All incubation was carried out at 37° C. Bacto-Tetrathionate Broth was used as the enrichment medium from which, after 24 hours' incubation, streak inoculations were made on Bacto-SS Agar plates. These were incubated and examined at 24 and again at 48 hr. for the presence of colourless colonies, which were fished to Bacto-Kligler Iron Agar slants. This medium made it possible to discard an occasional lactose fermenter that had not been clearly differentiated by the SS medium and also a type that failed to produce acid or gas from dextrose. Another complicating organism, which appeared occasionally, proved to be a slow lactose fermenter. It invariably fermented sucrose and was easily eliminated in this way. Organisms were classified in the *Salmonella* group when they satisfied the criteria outlined by Gibbons and Moore (5).

The exteriors of an additional 400 diseased eggs were examined for *Salmonella* types. Using aseptic precautions the eggs were washed with Bacto-Tetrathionate Broth, which was incubated and streaked on Bacto-SS Agar plates according to the method already described.

Because of the divergence of opinions expressed regarding any possible relation between bacterial content of eggs and season of year (8, 12), it was decided to ignore this factor and examine the diseased and normal series of eggs consecutively rather than concurrently, thereby obviating all possibility of doubt as to the origin of the cultures. The contents of the diseased eggs were cultured during July, August, and September; the normal eggs, during

* *Personal communication, 1944.*

October, November, and December; while the exteriors of the diseased eggs were examined at intervals during January, February, March, and April.

Results and Discussion

The bacteriological examination of 1000 eggs laid by a flock of pullorum reactors resulted in the isolation of *Salmonella* organisms from 61 of these eggs. Since these organisms were non-motile, failed to ferment maltose or dulcitol, and gave typical serological reactions, they were classified as *S. pullorum*. Of interest was the fact that 35 of these cultures, though apparently anaerogenic in dextrose broth, did show slight gas production in the butt of Bacto-Kligler Iron slants. The exteriors of 400 undamaged eggs from the reactor group were also cultured for the presence of *Salmonella* types. The media employed, although similar to those used in the examination of egg contents, failed to demonstrate the presence of organisms of the *Salmonella* group. The contents of 1000 eggs from a negative flock were also examined and were found to be free of *Salmonella* types according to the methods employed.

The bacteriological examination of 2400 hens' eggs resulted in failure to isolate any *Salmonella* types other than *S. pullorum*. The latter organism, perhaps the most difficult of all *Salmonella* types to isolate (3), was found in the contents of 6.1% of the diseased eggs, an incidence similar to that reported by a number of other workers (1, 4, 7, 11, 16). This is considered an indication that the methods used were sound and would have been suitable for the isolation of other *Salmonella* types had they been present. Watch was maintained for the possible occurrence of *Shigella gallinarum*, but it did not appear during the course of this survey.

It would appear from these results, which seem in accord with the literature on the subject, that hens' eggs are not likely to be infected with *Salmonella* types other than *S. pullorum*, though it must be admitted that failure to isolate other *Salmonella* organisms in a survey as limited as this does not necessarily indicate that such infections cannot occur. It is suggested that the evidence now obtained against fowl so closely akin to the domestic hen warrants continued study of the problem, with attention being directed towards the manner in which some of these other *Salmonella* types affect the laying hen.

Acknowledgment

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THE CROSS-VENTILATED TUNNEL SMOKEHOUSE[1]

By E. P. Linton[2] and A. L. Wood[3]

Abstract

A tunnel smokehouse with an output of 3000 lb. of smoked fillets per nine hour day has been in operation about one year. The temperature, relative humidity, and smoke velocity are controlled at optimum values independent of climatic conditions. Hence the colour and shrinkage of the product may be standardized and losses from cooking and dropping of the fish avoided. The relatively short time of smoking of two to three hours reduces spoilage of the fish to a minimum with resulting improvement in quality. Power and steam consumption have been kept as low as feasible.

During the past two years experiments have been conducted into the controlled smoking of fish, and as a result of these investigations an apparatus has been put into successful operation. This equipment may be of interest not only to the fisheries engineer but to technologists concerned with the smoking or drying of food products in general.

The primary purpose of the smoke curing process as carried out along the Canadian Atlantic seaboard is to impart to the fish a pleasant salt and smoke flavour and a golden yellow colour. The greater part of the smoked fish production consists of mild cured cod fillets, finnan haddie, and kippered herring, which are sold in the Canadian and United States markets. These mild cured products are perishable and must be handled in the same manner as fresh fillets. Hard cured herring which do not require refrigeration are also prepared in quantity for the West Indian trade. The smokehouse described here has been developed primarily for preparation of the mild cured products.

The procedure for the smoke curing of fish has not changed greatly in the last 50 years (2, 3, 8). The process is carried out in three stages—brining, drying, and smoking. The problems connected with the brining and drying of fresh fish previous to smoking have been discussed by Cooper and Linton (1) and the data on the physical conditions suitable for smoking, which have been collected at this Station over a period of years, mainly by Cooper and Linton, have been published only in the reports to the trade. A review of fish smoking has been published by LeGall (5). Lemon (6) has described an experimental smokehouse but no data are given on smoking conditions. Cutting (4) has discussed the engineering problems in the smoke curing of fish in England. He has developed a kiln primarily for the preparation of English kippers which, because smoking is used as a means of preservation,

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Contribution from the Atlantic Fisheries Experimental Station, Fisheries Research Board of Canada, Halifax, N.S.

[2] *Formerly Assistant Chemist, Fisheries Research Board of Canada; now Professor of Chemistry, Acadia University, Wolfville, N.S.*

[3] *Assistant Engineer, Fisheries Research Board of Canada.*

are both dried and smoked to a greater extent than the kippered herring sold on the Canadian market.

High temperatures during warm weather (resulting in soft partially cooked fish), high humidity, unevenness of smoking, dust particles on the fish, and excessively high labour costs are some of the faults in the smoking process as now carried on. Suitable smoking conditions may be summarized as follows: temperature of fish must not rise above 75° F., the relative humidity (usually about 65 to 70% at 75° F.) regulated to give required weight loss during the smoking period, velocity of smoke about 400 ft. per min., depending on the required length of the smoking period. Smoke density or smoke concentration will be discussed in a later article.

The Design of the Smokehouse

Any one of the numerous types of tunnel or compartment dryers might be adapted for smoking fish. The most important factor to be taken into consideration is that the air and smoke flow must be uniform, that is, they must have the same velocity throughout the cross-section of the smoking space in order to produce the same colour on all the fish. At the same time it is desirable to keep the power consumed by the fans to a minimum, as in many cases these smokehouses must be installed in outlying districts where power rates are high and the power available may be limited. Taking these factors into consideration, it appeared probable that the cross-ventilated tunnel type should give reasonably uniform smoke flow with a minimum consumption of power. Because of the difficulty of predicting air flow behaviour, it was necessary to construct a full scale commercial model. The tunnel smokehouse shown in Fig. 1 is constructed mainly of wood, is double walled, and finished with $\frac{3}{4}$ in. sheathing for use where insulated installations are required. Plywood, sheet metal, or wallboard are equally satisfactory. The size of the house is determined largely by the weight of fish that can be conveniently handled on the cars. The house holds two cars each carrying about 400 lb. of cod fillets. As the smoking and drying power of the smoke stream fall off noticeably in six to eight feet of travel over the fish, this distance determines to some extent the length of the cars. For instance, four cars cannot be used in the same equipment without an intermediate addition of heat and smoke. For convenience of the operator the smokehouse is maintained at slightly less than atmospheric pressure by the exhaust fan and hence it must be well constructed, all the joints must be sealed, and doors must be reasonably smoke tight.

Mechanical Equipment

The recirculating fan used in the present smokehouse is a reversible 42-in. propeller type fan. The fan is supported on a steel shaft that protrudes through one end of the smokehouse so that the driving motor is not operated within the smoke. When construction was started it was not known which was the most desirable direction in which to have the air and smoke flow

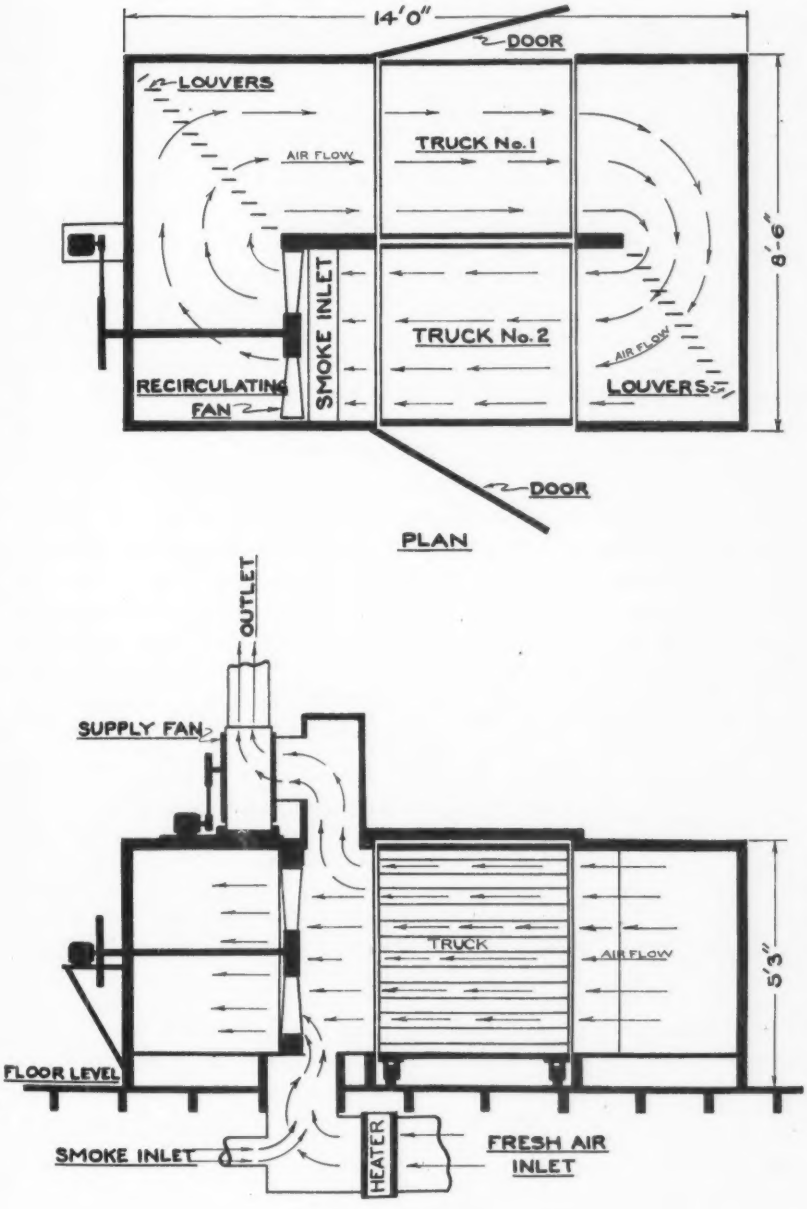

FIG. 1. *The cross-ventilated tunnel smokehouse.*

within the house, and, hence, the reversible type fan wheel was installed. The diameter of the fan was shown to correspond roughly with the height and width of the cars holding the fish. In the preliminary experiments the fan was driven by a 2 hp. motor using a speed reducer to give fan speeds varying from 135 to 470 r.p.m. After the preliminary experiments a $\frac{1}{2}$ hp. motor was connected to the fan with a V-belt. About 6000 cu. ft. of air per min. was recirculated by the fan.

The exhaust fan is a Clarage No. $1\frac{1}{2}$ driven at 990 r.p.m. by a $\frac{3}{4}$ hp. motor. A manually operated damper installed on the inlet side of the exhaust fan controls the volume of air vented to the atmosphere.

The steam heater installed on the inlet duct heats the incoming air sufficiently to maintain the smokehouse at the desired dry-bulb temperature. The dry-bulb thermostat installed in one end of the smokehouse controls the steam supplied to the heater and, hence, the temperature to which the incoming air is heated. Temperature control is maintained within 1° or 2° F. Both the dry-bulb temperature and wet-bulb temperature are recorded automatically with Brown psychrometers.

Smoke Producers

The type of smoke produced and the flavour of the fish are determined by the kind of sawdust burned, the water content of the sawdust, and the ratio of sawdust to air. For the most part, sawdust was purchased from local fish companies. It was free from strong odours such as those given off by fir or pine. The optimum water content of the sawdust was about 40%. The particle size varied from 0.1 to 0.2 in. in diameter.

Two types of burner were used. The first, a metal cone approximately six feet in height and five feet in diameter, placed over the ordinary planer-shavings sawdust fire, permitted a large volume of air to be drawn over the sawdust, giving a smoke very similar in odour and flavour to that used by the trade. This type of smoker is used commercially on installations of smoking equipment now in operation. The main disadvantage of the cone smoker is due to the inability to cool or dehumidify the air entering the smoker during warm weather. A fairly steady continuous smoke production is maintained by keeping two in constant operation, charging and relighting them alternately.

The second smoke producing equipment utilized was the Buffalo Smokemaster (John E. Smith's Sons Co., Buffalo, N.Y.). It has been used for the smoking of meats. It was found to require considerable attention and in general is not, in our opinion, as satisfactory as the cone smoker.

Smoke Density Meter

A simple type of smoke density meter was constructed with the equipment readily available. As a source of light, a 150 watt, 110 v. Mazda Projector Spot Lamp was installed in one end of the smokehouse. The path of light passed through about 40 inches of the smoke stream and then through a glass window to a Weston Photronic Foot-Candle Meter. Unaccountable variations in the intensity of the light from the Spot lamp were noted even though

a constant voltage transformer was installed in the circuit. Finally, fluctuations in the light sources were compensated for by standardizing the Weston cell meter with a 60 watt Mazda lamp installed outside the smokehouse near the Weston cell. The Weston cell was mounted on a pivoted base at a distance from the two light sources so that when swung from one to the other the cell gave the same zero reading of 200 fc. A variable voltage transformer connected in parallel with the two lamps enabled the zero reading to be set at any desired value. In measuring the smoke density, the meter was set at 200 fc. facing the 60 watt bulb and then swung to give the reading with smoke present. Most readings with smoke fell between 25 and 150 fc. but occasionally readings as low as 1 or 2 fc. were obtained. Optical density was calculated in the usual way by subtracting the logarithm of the reading with smoke present from the logarithm of the reading with no smoke in the house. The smoke density readings recorded are average values.

Temperature and Relative Humidity Control

The point at which smoke and air were admitted to the house is important, and several trials were made before the inlet and outlet were satisfactorily located. It was necessary to have the incoming fresh smoke and air mix with the recirculated smoke by passing through the recirculating fan. In this way uniform smoke and temperature distribution could be obtained across the section of the tunnel. Other points of inlet for the smoke were not satisfactory. Exhausting the smoke at a place shown in the diagram ensures that its temperature will be at a minimum and the relative humidity at a maximum, giving good economy of operation.

During the warm summer months when the dew-point exceeds 55° F. it is necessary to cool and dehumidify the fresh air entering the smokehouse along with the smoke. The smoke entering the house must not be cooled directly with ice or cooling coils as tars and smoke flavours, essential to the smoking process, are removed by this treatment. The only way to control the temperature (dry-bulb) and the relative humidity of the house is to admit a sufficient quantity of dehumidified and cooled air along with the smoke. The cooling and dehumidification may be carried out by mechanical refrigeration or by ice. In the arrangement used, the fresh air entering the house is drawn through an ice box containing 600 to 700 lb. of ice, crushed to give lumps 6 to 10 in. in diameter. This air is at a temperature of about 40° F. and is saturated with water vapour. It must then be heated by the steam heater to give the desired temperature within the smokehouse. Under the most adverse weather conditions likely to be met with in the Maritimes, about 1200 lb. of ice and 200 lb. of steam per hour will be consumed with the present equipment. The relative humidity of the smokehouse depends largely upon the volume of conditioned air mixed with the smoke entering the house. When the house is filled with fish, about 1500 cu. ft. of air per min. are mixed with about 300 cu. ft. of smoke per min. from the cone smoker.

Fish Supports

In most commercial houses the fish are supported on rods or hooks. The hanging of the fish is time consuming, and fish are often spoiled by dropping to the floor of the house. These difficulties are overcome by supporting the fish on wire-mesh trays.

The tray of most convenient size for the majority of fish products is about $2\frac{1}{2}$ by 4 ft. and consists of a metal screen supported by a welded steel frame. When trays are used to support the fish, the marks of the wire are quite noticeable on the fish. Fillets are placed with the skin side down on the smoking trays, while herring are placed with the cut side next to the screen. Fish are sometimes dyed with harmless dyes, to increase the colour. On such fish the marks of the trays are scarcely noticeable. The loading capacity of the tunnel depends primarily on the spacing of the trays on the cars and on the size of the fish being smoked. For cod fillets the trays should be placed one above another about $3\frac{1}{2}$ in. apart. Large steak cod will give a loading of about 4 lb. per sq. ft. of tray area. With market cod, loading is about 2 to $2\frac{1}{2}$ lb. per sq. ft. and with scrod, about $1\frac{1}{2}$ lb. Fillets are loaded on the trays with their length in the same direction as the air flow. With kippers the trays may be placed closer together on the cars and the trays are loaded to about $1\frac{1}{4}$ lb. per square foot. Finnan haddie give about the same loading per square foot as cod fillets. Although the fish may be hung on rods that are supported on the cars in somewhat the same manner as in the present commercial houses, the loading capacity of the tunnel smokehouse will be reduced by about one-third. Furthermore, considerably more labour is required for hanging the fish on rods than simply placing them on the trays.

When the trays are used day after day for the smoking of fish, a considerable deposit of smoke tars and particles of fish accumulate on them. Furthermore, they tend to rust and may leave undesirable rust marks on the fish. These difficulties are best avoided by waxing them periodically. When the trays are new they are dipped in hot paraffin wax and allowed to drain. Other waxes, such as Opalwax, manufactured by Canadian Industries Limited, which melts at 176° F. may be used, or an aqueous suspension of Johnson's Clear Rust Inhibiting Wax No. 1568 may be applied directly to the trays. The ordinary paraffin wax appears to be the most economical of those tried.

Air Flow and Power Consumption

Studies were made on the air flow to determine the best direction of flow and the most suitable means of ensuring even distribution across the cars.

When the air and smoke were blown directly onto the first car of fish a dead air space in front of the hub of the recirculating fan was formed. Also the rotating blades collected tar and soot, later throwing them onto the fish. Therefore the rotation of the fan was reversed. This changed flow permitted the thorough mixing of the incoming smoke with that being recirculated.

Three different types of devices for straightening the air flow were investigated. The results of air flow measurements and wattage consumed for each device are shown in Table I. Semicircular splitters were installed in the first trials. As the table shows, they are quite efficient and possibly require slightly less horsepower than the other devices used. However, as they are

TABLE I

WATTS REQUIRED BY DIFFERENT TYPES OF AIR FLOW STRAIGHTENERS

Fan, r.p.m.	Semicircular splitters		Wooden louvers		Grids	
	Air velocity, ft./min.	Watts	Air velocity, ft./min.	Watts	Air velocity, ft./min.	Watts
135	325	120	350	30	200	70
260	650	270	600	170	375	270
326	900	430	770	350	450	470
400	1100	760	1000	680	600	860
470	1300	1200	1100	1120	725	1330

rather difficult to install and construct, they were abandoned in favour of the wooden louvers. The wooden louvers were 4 in. wide and $\frac{1}{2}$ in. thick and were set on pivots four inches apart so that they could be adjusted to direct the air flow where required. This device is simple to construct and gives uniformity of air flow. A third trial was made with an arrangement of grids constructed of wallboard. The grids were about 3 in. wide and were more or less uniformly spaced across the face of the tunnel, the spacing arranged to give uniformity of air flow. It was found practically impossible to avoid dead air spaces, and furthermore, as the table shows, the wattage consumed for a given air velocity was considerably greater than with either the semicircular splitters or the wooden louvers. With wooden louvers installed as shown at both ends of the smokehouse, the air velocity was found to range from 400 to 500 ft. per min. across the section of the house where the fish are placed. The variation of 100 ft. per min. is not considered to be significant in view of the fact that the cars pass periodically through the house. At this air velocity $\frac{1}{2}$ hp. was consumed by the recirculating fan.

In an effort to keep the house to a minimum size, experiments were made to shorten it by erecting false walls at various positions in the two ends of the house. It was found necessary to have the wall at the inlet end about $3\frac{1}{2}$ ft. from the fan, and at the other end about 40 in. from the cars of fish in order to obtain satisfactory air flow.

The exhaust fan is capable of handling about 2000 cu. ft. of air per min. against a one inch static pressure. About 390 watts is required by the motor operating this fan.

The Experimental Smoking of Fish

Smoking experiments carried out in the tunnel smokehouse, using various types of fish and smoking conditions, have demonstrated the uniformity of smoke flow in the house, and have established the effect of some of the factors concerning the smoking, such as pre-drying and smoke velocity. The results are given in part below and in part by Linton and French (7).

The uniformity of air flow has considerable influence on the evenness of drying and smoking. In a typical experiment 800 lb. of cod fillets, obtained from a local fish company, were smoked for two and one-half hours, the dry-bulb temperature being 76° F., and the relative humidity, 70%. The cone smoker was used as the source of smoke and about 1000 to 1500 cu. ft. of air and smoke were exhausted from the tunnel per minute. Trays of the fish at various points in the trucks were weighed before and after smoking. The average weight loss of the cod fillets was 11.4% and the extreme range of weight loss in the weighed trays of fish was from 12.2 to 10.5%. In view of the fact that the fillets were not of the same size, this variation is not large enough to be of any importance. Furthermore, on examination the next morning, the variations in the colour of the fillets were scarcely noticeable. The fish were packed for sale by a local company and all of them were packed out as first quality. In commercial practice, fish are sorted into three sizes before smoking, as fish of different sizes smoke and dry to a different degree under the same smoking conditions. With this sizing, differences in colour would not be detectable.

In the operation of the present commercial smokehouse it is customary to allow the fish to drain an hour or so, and then dry for one or two hours over open wood fires until the surface is partially dried and the fish are sticky to the touch. The weight loss with cod fillets is usually 5 to 7% during this pre-drying. Pre-drying is considered to be an essential part of the smoking procedure in order that a glossy surface may be formed on the fish. The pre-drying may be carried out in the present tunnel smokehouse in 40 to 60 min., but it is also possible owing to the higher smoke velocity used in the tunnel smokehouse to omit the pre-drying step for fillets and smoke them after they have drained for a few minutes. An attempt was made to determine whether there was any appreciable difference between the appearance of fillets that had been pre-dried and those that had been smoked without preliminary drying. Eight hundred pounds of market size cod fillets were loaded on the two cars and the fish in one car were dried for 50 min., resulting in a weight loss of 4 to 5%. Both cars were then smoked for three hours at a dry-bulb temperature of 75° F. and about 73% relative humidity. On packing out the fish from the cars there was very little difference, if any, in the surface appearance of those processed by the two different methods. Obviously there was a slightly higher weight loss of 1 to 2% in the fillets that had been pre-dried. From this experiment it was concluded that with a thin smoke from the cone smoker (average density, 0.20), it is not necessary to pre-dry fillets previous to smoking. In some cases, where a relatively

large weight loss is desired it may be necessary to pre-dry the fish. The pre-dried fish do not have as deep a colour for the same time of smoking as the undried fish.

In the majority of the fish smoking experiments the velocity was set at about 450 ft. per min. Using this smoke velocity, the power was maintained at a value that could be drawn from local lighting circuits, and furthermore, it gave a considerable decrease in the smoking time over the present commercial methods. As indicated above, sufficient colour was obtained in the cod fillets in two to three hours at 450 ft. per min. smoke velocity. In one experiment smoke velocity was decreased to 125 ft. per min. and this lengthened the smoking period to five to six hours to give the same depth of colour, other conditions being the same.

Other species of fish—herring, haddock, and mackerel—have been smoked in the house with very good results. In commercial practice these fish are hung from hooks and this may be done in the present type of smokehouse. However, in our experiment they were laid flat on the metal trays. Herring are placed cut side down on the metal surface, so that when packed, the tray marks do not show on the skin surface. Kippered herring acquire sufficient colour in about three hours and lose about 15% in weight at a relative humidity of 65% and a smoke velocity of 450 ft. per min. in the smoking period.

Several experiments were carried out on the smoking of herring fillets for canning purposes. The fillets are laid skin side down on the trays and acquire sufficient smoke in 40 to 60 min. to give the desirable golden yellow colour on the skin of the fillet. They may be dried to any extent by controlling the relative humidity within the house, by the admission of conditioned air or, if necessary, by pre-drying.

In continuous operation the cars of fish are moved through the house at time intervals equal to one-half of the required smoking period. As each car remains in each position in the smokehouse for one-half of the smoking time, uneven air flow in one position is largely compensated for in the second position. Furthermore, the direction of smoke flow with respect to fish is reversed on moving the cars from one position to the other and all sides of the fish are smoked to the same extent. Increased capacity is obtained by installing several units side by side so that each car runs through one, two, or more units as required.

This method of smoking fish is essentially continuous as contrasted with the batch operation of the present commercial houses. This is a considerable advantage in lowering labour costs. Furthermore the fish do not deteriorate in quality to such an extent during the greatly shortened time of smoking.

The tunnel smokehouse has been in operation for about a year, and no serious defects have become apparent. One objection, the importance of which cannot be forecast definitely, lies in the fact that the development of colour is rather a slow reaction, maximum colour being reached after one to three days, depending on the temperature. Hence it is somewhat difficult for the operator to determine when the fish are sufficiently smoked.

On the other hand, it approximates continuous operation, cars being removed in little less than hourly intervals. Because of the very materially increased smoking rate and of the greater capacity of the effective smoking space, the output per cubic foot per hour is of the order of 50 times that of the regular smokehouse. Its construction is inexpensive and its operation is simple. The equipment should be of definite assistance to the producers and processors of smoked fish.

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A COMPACT CARBON PILE RHEOSTAT[1]

J. T. BURT-GERRANS[2]

Abstract

This paper describes a carbon compression rheostat having, in addition to all the advantages set out in the handbooks of electricity and engineering, compactness and great steadiness of operation. The method of operation is detailed and a graph of a performance test is included.

Introduction

General discussions of carbon compression rheostats with citations of advantages and fields of application are published in handbooks of electricity and engineering, e.g., Marks (1, p. 1593), Pender (2, pp. 1233-1234), and Pender and Del Mar (3, 4–08), but, in the literature, descriptions of particular forms are few. The rheostat herein described was developed in Toronto for use in electric furnace work about twenty-five or more years ago and has been used successfully, as occasion arose, ever since. Rheostats based on this pattern have been in use also in the laboratories of the National Research Council, Ottawa, for upwards of 10 years.

The apparatus is particularly adapted to low voltage electrolytic work, because of the very fine stepless adjustments obtainable. With it, currents up to 1200 amp. may be continuously varied and controlled by the turning of a threaded brass or copper pressure rod. Although the rheostat works best on low voltages, it may be used on any voltage up to 110 a.c. or on the same high d.c., provided that the cooling water is neither hard nor sufficiently loaded with purifying or softening chemicals to render it conducting. Most industrial waters, however, are suitable.

Description

In Figs. 1 and 2 it may be seen that a standard asbestos pipe 4 in. I.D. × 24 in. long is filled with carbon plates $\frac{1}{4}$ by 3 by 3 in., cut into U-shape* and stacked as shown in plan (Fig. 1, C). This arrangement provides a central vertical channel, a slot in each side of each plate and eight periferal vertical channels for the cooling water. The whole, together with two brass pressure equalizer plates, is held between two flanged and gasketed brass castings or turned plates with lugs (Fig. 1, B), with six $\frac{1}{4}$ in. iron tie rods. The upper equalizer plate is $\frac{3}{8}$ by 3 by 3 in., and the lower $\frac{3}{8}$ by $3\frac{7}{8}$ in. in diameter, centre bored with a 1 in. hole. The tie rods are insulated from the castings by means of rubber bushings and fibre washers. The gaskets, shown only in

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Contribution from the Department of Electrochemistry, University of Toronto, Toronto, Ont.

[2] *Professor.*

* *All plates were obtained from the Canadian National Carbon Company, Toronto, Canada, those lately supplied being ready cut.*

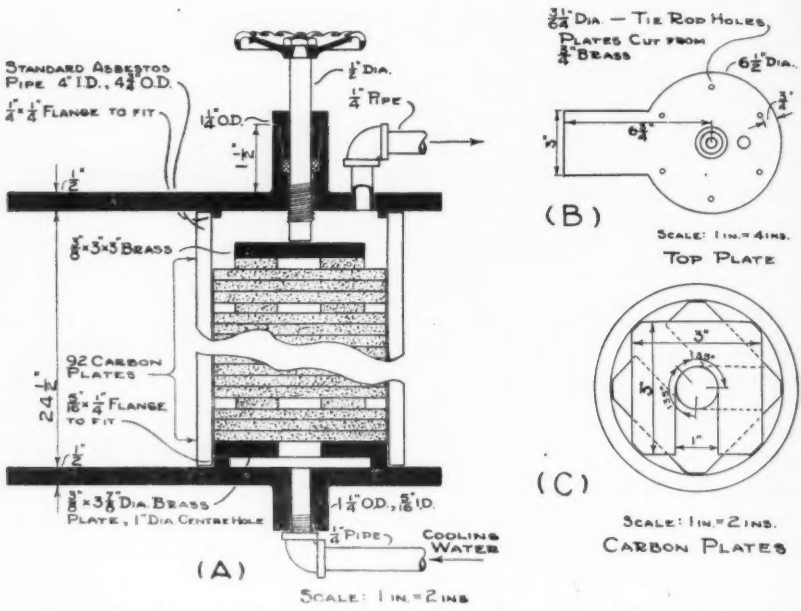

FIG. 1. *(A) Vertical section. (B) Casting detail, plan. (C) Arrangement of carbon plates, plan.*

Fig. 2, are of rubber, or steam packing, and seal the joints between pipe and castings. The packing gland (Fig. 1, A) prevents water leakage around the pressure rod. Electrical connections are clamped or bolted to the lugs and water connections made with ½ in. rubber garden hose.

Operation

In operation, water enters at the bottom, passes upward in the central channel through the slots into the vertical periferal channels and out through the top. The flow should be about 15 litres per minute, depending on the amount of power to be absorbed. This should be the first adjustment. With the pressure rod loosened to its limit, the rest of the circuit is completed and then the rod screwed down until it touches the plates. Thereafter, the change in pressure on the plates caused by the slightest turning of the rod is indicated by an ammeter in the circuit. Only one complete turn of the rod is required to cover the whole range of pressure. When the rheostat is not in use the pressure should always be released, otherwise the plates are apt to stick together. Though considerable pressure may be applied safely, it is possible to break the plates by screwing them down too tightly; this should be kept in mind. When the applied voltage is 50 or more, currents below 50 amp. are apt to be unsteady because of the low pressure required. Below 50 v., however, the currents are quite steady.

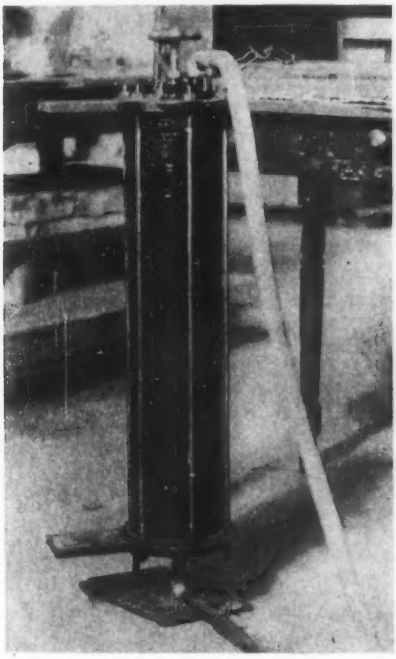

FIG. 2. *Rheostat assembled.*

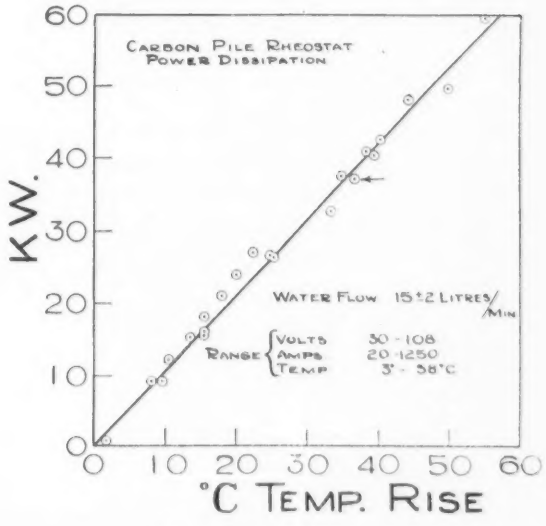

FIG. 3. *Graph of performance.*

Performance

Fig. 3 is a graph of the results of a series of measurements made when the rheostat was connected to a 25 cycle a.c. transformer, variable between 30 and 120 v. in 3 and 6 v. steps. The line represents the theoretical kw. values for a flow of 15 litres per minute: it has a slope of 1 kw. per degree rise in temperature of the water. The point marked with an arrow represents the observed value for 1250 amp. at 30 v.

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RESIN-RUBBER FROM CANADIAN GROWN PLANTS

IV. ANALYTICAL STUDY OF MILKWEED POD GUM[1]

By R. W. Watson[2] and N. Levitin[3]

Abstract

Fresh pod gum has an acetone-soluble content (resin) of 83.4%, and a benzene-soluble content of 7.3%. Steam distillation of the resin furnishes a small amount (0.1%) of an essential oil. Acidification of the saponifiable portion (55%) of the resin yields an oil consisting of a mixture of fatty acids. Palmitic, stearic, oleic, linoleic, and linolenic acids were identified. Resin acids are absent. The unsaponifiable portion (45%) contains a large amount of α-amyrin, and extremely small amounts of sterol (probably heterogeneous) and resene. Polyisoprene, an oil containing free acids, and an unsaponifiable residue make up the benzene extract. Water-solubles include calcium tartrate, soaps, and a material yielding a mixture of sugars on hydrolysis. The residue comprises lignin, cellulose, protein, and inorganic materials. From these data the dry gum contains approximately 50% of fatty acid esters, 26% of α-amyrin, 6% of polyisoprene, 4% of ash, 3% of lignin, 2% of cellulose, and small amounts of protein, sterol, resene, essential oil, and calcium tartrate. Unidentified substances comprise 7.7%.

Introduction

Recent interest in the production of milkweed floss has led to a study of possible by-products from the waste hulls. The following paper in this series will deal with the extraction of a resin–rubber gum from alkali-digested pod hulls of the common milkweed, *Asclepias syriaca* L. (20). Little is known concerning the chemical composition of the raw material, and the gum itself has not hitherto been investigated.

Besides the fruit walls a varying proportion of seeds, floss, and placentae occurs in the material from which the gum was mechanically extracted. Rheineck (17) in 1939 reported the first chemical investigation of the follicle walls from which an ester (believed to be the propionate) of α-amyrin, and a hydrocarbon ($C_{29}H_{52}$; m.p. 63° C.) were identified. The presence of a polymerized hydrocarbon $(C_5H_8)_n$, assumed to be the *cis*-configuration (rubber) of polyisoprene, was also reported. It has since been shown that the polymer present in the stem-latex of milkweed is true rubber (14). Linoleic acid has been identified, and a sterol has been isolated and characterized from the oil extracted from milkweed seeds by petroleum ether (17). Rheineck has also isolated a sterol from the seed hairs. So far no chemical study of the placentae seems to have been made.

Experimental and Discussion

Preliminary acetone extractions, drying of the residues, and subsequent benzene extractions of small samples (4 gm.) of dry gum were conducted in

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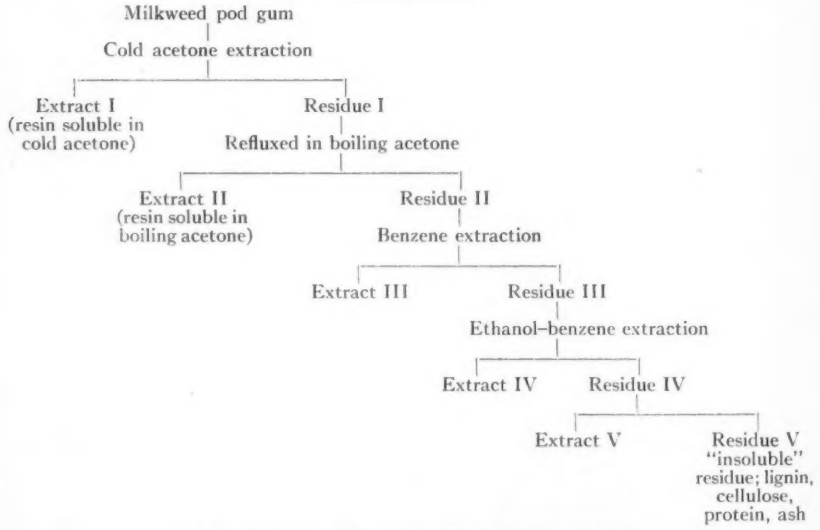
[2] *Biochemist, Industrial Utilization Investigations.*

[3] *Senior Laboratory Assistant, Industrial Utilization Investigations.*

Soxhlets, to which clean flasks with fresh solvent were attached at intervals. Hyperbolic extraction curves were obtained, becoming asymptotic for acetone at about 200 hr., and for subsequent benzene extraction, at about 100 hr. In this way at least 83.4% of the gum was found to be acetone-soluble (resin) and 7.3% benzene-soluble.

The resin for this investigation was obtained by thorough extraction with (*a*) cold acetone and (*b*) boiling acetone of about 1540 gm. of fresh gum containing 20.3% of water. To avoid prolonged heating of the extracts in the boiling flask, fresh solvent was substituted at intervals. The residue from the cold acetone extraction was refluxed for 16 hr. with boiling acetone, and then extracted successively in large Soxhlets with benzene, azeotropic ethanol–benzene, and water. The solvent fractionation of the gum is outlined, and the extracts and residues numbered in Diagram I.

DIAGRAM I



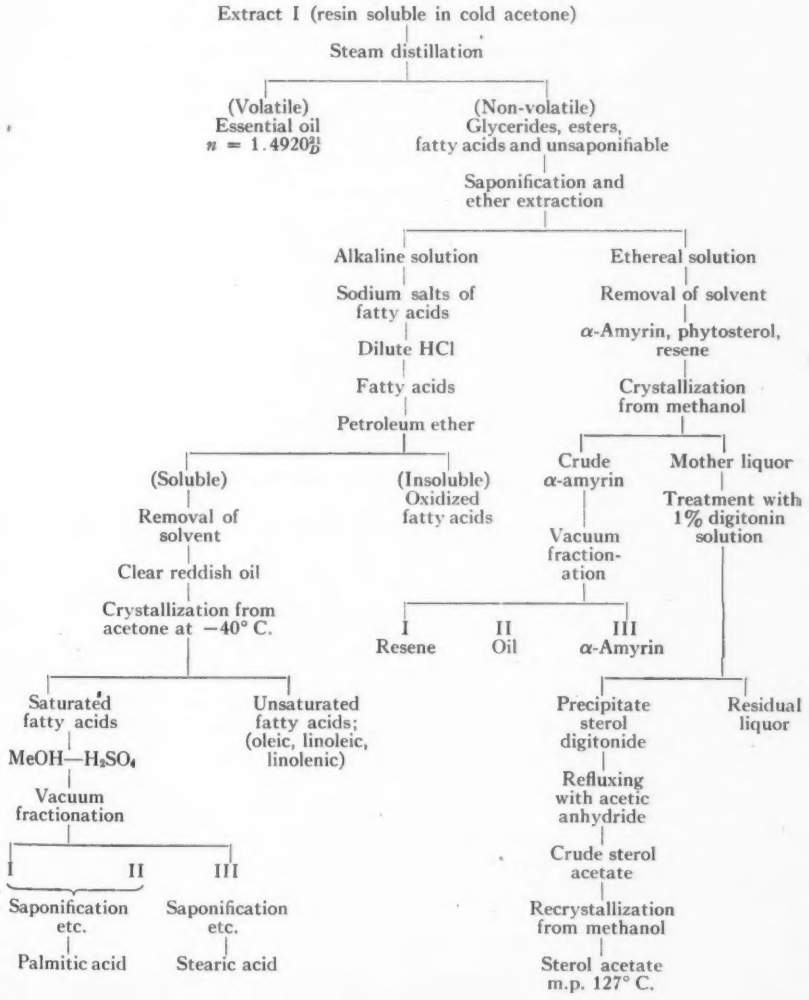
Cold Acetone Extract I (see Diagram I)

The cold acetone extracts were combined, the solvent recovered under partial vacuum (nitrogen), and the residue dissolved in ether. After washing to neutrality with water the ethereal solution was dried over sodium sulphate and Drierite. Water soluble material in the resin is negligible. Distillation of the ether yielded a soft yellow resin, with an iodine absorption value of 95.2*, an acid value of 10.5, and a saponification value of 139.3. Extraction with several portions of 5% aqueous sodium bicarbonate, combination of the extracts, acidification and re-extraction with ether yielded 0.17% of colourless semisolid fatty material.

**All iodine numbers were determined by the Wijs method.*

About 160 gm. of the neutral cold acetone extract, dispersed in hot water and subjected to steam distillation for seven hours, yielded about 1500 ml. of a slightly milky distillate. After shaking the distillate with ether, and drying the ethereal solution over sodium sulphate, a small amount (0.2 gm.) of a yellowish brown oil ($n = 1.4920^{21}_{D}$) was obtained on recovery of the solvent. This oil possessed a strong characteristic odour, and was present to the extent of about 0.1% in the gum. The further separation of the cold acetone extract into groups of substances is outlined in Diagram II.

DIAGRAM II



Saponification of Esters

The residual resin after steam distillation consisted of a mixture of glycerides or other esters of fatty acids, α-amyrin, and sterol, along with resene and unidentified resinous substances. The saponifiable fraction (55%), a brown turbid oil, was quantitatively determined by the modified Kerr–Sorber method (7). To obtain a quantity of this oil, about 157 gm. of washed dry resin was dissolved in enough 95% ethanol to make a 25% solution, and refluxed with potassium ethanolate for one hour. After rapid cooling, the mixture was diluted with ether in a large separatory funnel. On extracting with several portions of 0.2 *N* aqueous potassium hydroxide, filtering, and acidifying with hydrochloric acid, a considerable quantity of the same turbid oil was obtained. Extraction with ether and drying in the usual way yielded 91.1 gm. (58%). A light brown solid settled out on standing.

The ethereal solution of unsaponifiable substances was washed to neutrality with water, and dried over sodium sulphate, and Drierite. Removal of the ether left a cream-coloured plaster-like solid, comprising 42% of the resin.

The fatty acid oil was clarified by taking it up in sufficient light petroleum ether to make a 10% solution, and filtering. This treatment removed 5.5% of some insoluble material, which was not further investigated. The filtrate was refluxed with activated charcoal, filtered, and shaken at intervals for several days with diatomaceous earth. This procedure failed to remove the colouring matter, and on recovery of the solvent, a clear reddish oil with the characteristic odour of the essential oil remained. The constants of this fatty acid oil are given in Table I.

TABLE I

CONSTANTS AND COMPOSITION OF MILKWEED OILS

	Seed oil	Pod gum oil
Acid value	11.0	184.4
Hexabromide value	0.0	3.2
Iodine value	122.8	112.5
Refractive index	1.4730^{25}_{D}	1.4729^{25}_{D}
Saponification value	191.9	191.3
Specific gravity	0.9221^{25}_{25}	0.9177^{25}_{25}
Linoleic acid, %	43.8	35.4
Linolenic acid, %	0.0	2.8
Oleic acid, %	51.8	55.3
Saturated acids, %	4.4	6.5

The fruit walls of *A. syriaca*, according to Rheineck, contain a fixed oil, and several investigators (6, 10, 13) have reported the presence of about 20% of a semidrying oil in the seeds. The constants of the seed oil, as determined by Lanson, Habib, and Spoerri (10) are compared in Table I with those of the pod gum oil from this investigation. The similarity seems to indicate that the oil which forms such a large proportion of the gum may come from seeds present in the raw material.

Four separate esterifications with methanolic sulphuric acid (23) invariably failed to yield a measurable acidic residue. This observation, taken in conjunction with the amount and character of the material removed from the resin by 5% aqueous sodium bicarbonate, conclusively demonstrates the absence of resin acids. Addition to the oil of excess ether or ethanol yielded faintly turbid solutions from which a trace of a white solid separated on standing. On the addition of a small amount of 1% digitonin in 90% ethanol to a hot ethanolic solution of the oil, a few crystals of digitonin steride separated after several days. The presence of this trace of sterol is due in part to its solubility in unsaponified oil, and also to the inefficiency of extraction in large separatory funnels.

Separation and Identification of the Fatty Acids

Vacuum fractionation in a semimicro column (1) of the methyl esters of 1.3 gm. of saturated acids, separated by crystallization from the oil in acetone at −40° C. (4), yielded three fractions. Fraction 1 was a colourless oil (m.p. 17° C.)*. Fraction 2 consisted of a colourless crystalline solid (m.p. 22° C.). On combining Fractions 1 and 2, and saponifying with potassium alcoholate, the reconstituted acid, after recrystallization from acetone at −40° C., had a melting point of 62° C., and proved to be identical with palmitic acid. Calc. for $C_{15}H_{31}COOH$: C, 75.01; H, 12.50%. Found: C, 74.80; H, 12.42%. Fraction 3 was a light yellow, crystalline solid, which melted sharply at 39° C. After saponification, and regeneration of the acid, it was twice crystallized from acetone at −40° C., and once from 94.4% ethanol at 0° C. This yielded a small quantity of stearic acid, which melted at 70.6° C. Several times as much palmitic as stearic acid was present.

The residual acids from the separation of the saturated acids were recovered by removal of the acetone in an atmosphere of carbon dioxide, and taken up in ether. The hexabromide value (3) was found to be 3.25, corresponding to a linolenic acid content of 1.2%. Tetrabromostearic acid, crystallized from the brominated ethereal solution, after three crystallizations from petroleum ether, had a constant melting point of 114.1° C., thus proving the presence of linoleic acid. Dihydroxystearic acid, prepared according to the method of Hazura (5), and crystallized twice from ether, and once from ethanol, had a melting point of 116.1° C. Calc. for $C_{18}H_{36}O_4$: C, 68.36; H, 11.40%. Found: C, 68.35; H, 11.87%. The anomalous melting point was therefore due to mixture of the α- and β- forms, and the presence of oleic acid is established.

Composition of the Oil

The method of Brown and Stoner (2) as modified for soy bean oil by Earle and Milner (4) was used in the direct quantitative determination of the saturated fatty acids. Three crystallizations from acetone at −40° C. yielded 8.6% of a near-white solid, with an iodine absorption value of 12.8. Assuming

* *All melting points are corrected.*

the only remaining unsaturated acid to be oleic, the presence of 7.4% of saturated acids in the oil is indicated.

A preliminary attempt to determine the composition of the mixed fatty acids was made by the spectrophotometric method of Mitchell, Kraybill, and Zscheile (12). Triplicate determinations showed close agreement, and gave an average specific α of 32.7 at 2340 Å, corresponding to 37.7% of linoleic acid, and a specific α of 1.5 at the 2680 Å peak, proving the presence of linolenic acid (2.8%). Calculation from the iodine value (112.5) of the mixture, showed 40.7% of oleic acid and 18.8% of saturated acids by difference. The discrepancy in the values for the saturated acids drew attention to the possible presence of some impurity, soluble in acetone at $-40°$ C., and possessing low unsaturation.

A sample of the oil was further purified by conversion of the acids to the methyl esters, distillation, and reconstitution of the acids. About 11.5 gm. of the mixed acids was esterified and distilled (180° C. (0.02 mm.)) using tared flasks. A dark tarry residue, amounting to 13.9% of the original mixture, remained in the distillation flask. The regenerated oil was of a pale straw colour (iodine value 121.4), and, when analysed by the spectrophotometric method (12), gave the composition reported in Table I. The proportions of saturated acids revealed by the direct and indirect methods were now in fair agreement.

Unsaponifiables

After removal of the free fatty acids, glycerides, and much of the essential oil by saponification and steam distillation, the unsaponifiable residue contained α-amyrin, and one or more sterols and resenes, besides uncrystallizable resinous substances.

Isolation and Identification of α-Amyrin

An accurate method for determining the amyrin content of milkweed resin was developed. This procedure utilized prolonged vacuum sublimation (160° C. (0.1 mm.)) in a special apparatus to be described in a later report, and showed the presence of at least 69.53% of α-amyrin in the total unsaponifiable residue. The preparation of a considerable quantity of pure amyrin involved refluxing the unsaponifiable residue (52.2 gm.) in sufficient methanol to effect complete solution. Crystallization was complete after two weeks, and the crude amyrin was separated by filtration, washed, and dried. It was then subjected to vacuum sublimation, and the product fractionated by further sublimation *in vacuo*. The purest fraction melted at 184.3° C. Calc. for $C_{30}H_{49}OH$: C, 84.50; H, 11.74%. Found: C, 84.45; H, 11.74%. Mol. wt. 417 (16).

Fractional sublimation (160° C. (5 mm.)) of the crude amyrin obtained by a single crystallization from methanol yielded three fractions. Fraction 1 was a colourless oil, which was not further studied. Fraction 2 consisted of colourless acicular crystals, m.p. about 50° C., which were not identified owing to paucity of material. Fraction 3 comprised about 99% of the combined fractions and consisted of α-amyrin.

Isolation of Sterol

After repeated concentration and crystallization of α-amyrin from the mother liquor, the residual liquor was diluted with 95% ethanol, and any sterol present precipitated, after long standing, as the digitonide. A small quantity (0.75 gm.) of dry digitonin steride, corresponding to 0.3% of sterol in the unsaponifiable residue, was collected. The digitonide was refluxed for one hour with acetic anhydride, the mixture poured into excess water, and the sterol acetate twice crystallized from methanol. Its melting point was then constant and sharp at 127° C. This material gave positive tests with Whitby's colour reactions *A* and *B* (22). Calc. for $C_{27}H_{46}O_2$: C, 80.60; H, 11.45%. Found: C, 80.61; H, 11.43%. Attempts to obtain anything further of a definite nature from the residual mother liquor failed.

Sterols occur in most plants as mixtures of closely related compounds with similar solubilities, as shown by the work of Lobert (11), Thornton, Kraybill, and Mitchell (19), King and Ball (9), Holden (8), and others. The present sterol in all probability is also a mixture, and the attempt to characterize it through the acetate is merely provisional. Successive hot saponifications (20) had presumably reduced any amyrin or sterol esters in the original material to the corresponding alcohol.

Hot Acetone Extract II (see Diagram I)

From the material extracted by boiling acetone a small quantity of a fixed oil and resene were separated. Removal of acetone from the combined extracts by distillation under partial vacuum (nitrogen) left a small quantity (5.9 gm.) of a clear viscous resin with a strong odour of the essential oil. This extract represented 0.5% of the original weight of dry gum.

About half this entire quantity (2.8 gm.) was taken up in ether to make a 25% solution, and a small amount of suspended matter removed by filtration. The filtrate was extracted with several portions of 5% aqueous sodium bicarbonate. Acidification, extraction with ether, and removal of the solvent, left a small amount (0.14 gm.) of yellowish brown oil ($n = 1.4917^{25}_{D}$) with a strong odour of the essential oil.

Isolation of Resene

Further treatment of the remaining ethereal solution resulted in the separation of the resene. In view of the unknown structure of this compound, Tschirch and Stock's term is retained as most appropriate. Recovery of the ether, saponification, and extraction with dilute aqueous potassium hydroxide gave on acidification a considerable quantity (31.5%) of a viscous dark brown oil ($n = 1.4830^{25}_{D}$, iodine absorption value 142.5) with the odour of the essential oil. An insoluble fraction, which collected at the interface, yielded, on recovery of the solvent, a mixture (16.9%) of colourless waxy films imbedded in a resinous matrix. The waxy substance forming these films is the resene referred to below. Nothing further of a definite nature was obtained from this fraction.

The yellow ethereal solution of unsaponifiables left a clear brown resin on recovery of the solvent. Attempts to resolve this material failed.

Vacuum distillation (180° C. (0.005 mm.)) of a sample of the hot acetone extract yielded two fractions. Fraction 1 was a yellow mobile oil. Fraction 2 consisted of a translucent resene. The latter was recrystallized several times from methanol, from which it separated as shining scales (m.p. 66° C.). Owing to lack of sufficient material it was not subjected to elementary analysis.

Benzene Extract III (see Diagram I)

The gum fraction soluble in benzene (7.3%) included free acids, a fixed oil, polymerized hydrocarbon, and a resinous unsaponifiable residue containing resene. The extracts were removed at intervals, and fresh solvent was added in order to avoid prolonged exposure to high temperatures. After thorough extraction the various portions were combined and concentrated under partial vacuum.

A sample (100 ml.) of the concentrate was treated with several times its volume of cold acetone, whereupon a light brown precipitate of the polymer separated. Collected, washed and dried, this impure hydrocarbon weighed 7.3 gm. The dry residue from the filtrate was taken up in ether, and extracted in the usual way with 5% sodium bicarbonate solution, yielding a clear yellowish-brown oil ($n = 1.4816_D^{27.2}$) with a slight odour of the essential oil. After complete extraction of the free acids with ether, a yellow colouring matter remained in the aqueous phase. Part of this material was removed by shaking with chloroform, but it was not identified.

The hydrocarbon proved to be a low polymer. During saponification of the benzene extract and subsequent extraction with ether the residue that escaped collection after the addition of acetone was mechanically separated, washed, and dried (1.4 gm.). Evaporation of the ethereal solution of unsaponifiables left a soft brown resin, which was not further studied. The molecular weight of the polyisoprene was determined on a sample extracted from fresh gum (*a*) with 1% phenyl-β-naphthylamine in cold acetone, and (*b*) with 0.5% benzidine in nitrogen-saturated benzene. The material was not dried after acetone extraction. After precipitation from benzene it was dried to constant weight *in vacuo* at room temperature, and had a weight average molecular weight of about 19,000 (18).

Of the entire portion of the gum soluble in benzene, about 79% consisted therefore of impure polyisoprene, 15% was a viscous oil, and 6% was unsaponifiable matter. Solution of the unsaponifiables in ethanol left a small quantity of a colourless wax-like compound.

Ethanol–Benzene Extract IV (see Diagram I)

Small quantities of fixed oil and unidentified substances were obtained from this extract. A soft brown resin (2.6 gm. amounting to 0.2% of the original dry gum), of which a considerable fraction was insoluble in ether, was obtained

on removal of the solvents. When collected, the matter suspended in ether formed a black tarry mass, from which nothing definite was obtained. Extraction of the ethereal solution with 5% sodium bicarbonate solution yielded a small quantity (0.18 gm.) of a viscous yellow oil ($n = 1.4930^{25}_{D}$). Subsequent extraction with sodium hydroxide solution (5%) yielded an even smaller quantity (0.04 gm.) of an odourless yellow oil. The ethereal solution remained sharply divided into three layers, which were treated separately. Removal of the clear dark brown bottom layer, and recovery of the solvent, left a brown resin (0.5 gm.) with a strong odour of the essential oil. The insoluble substances from the middle layer dried to a powder that gave a positive test with Fehling's solution, and on acidification yielded a yellow oil. Recovery of the solvent from the top layer left a yellowish waxy material that was precipitated from dioxane in an amorphous condition, and that gradually charred without melting when heated slowly to 300° C.

Aqueous Extract V (see Diagram I)

Calcium tartrate, a dark tarry material yielding sugars after acid hydrolysis, soaps, and inorganic substances from the soluble ash components were found in the water extract. After seven consecutive extractions, each lasting for two days, there was no indication of an approaching end-point, and the extraction was discontinued. In this 14 day period, 10.7% of the original residue had been removed. The extracts, preserved under toluene, were filtered, and subsequently concentrated to a thin syrup.

The dry filter residue (5 gm.) was a light brown powder containing glistening scales and a small amount of calcium tartrate. A small amount (0.6 gm.) of this powder was boiled with 0.2 *N* potassium hydroxide solution, and the brown suspension filtered, acidified to pH 3, and extracted with ether. On re-extracting with 5% aqueous sodium bicarbonate, the entire extract passed to the aqueous phase. Removal of the ether left 0.06 gm. of a viscous yellow oil. About 10% of the filter residue therefore consists of free acids, held in the gum as soaps. Ignition of the material left after extraction with potassium hydroxide yielded 66% of ash, whereas the original filter residue contained 53.4% of ash.

The thin syrup (200 ml.) obtained by prolonged boiling of the filtrate (4 litres) was separated by filtration from masses of black tarry material and from a considerable inorganic residue containing calcium phosphate. The filtered liquor gave a positive test with Fehling's solution, but failed to form osazones. About 50 ml. of the filtrate was acidified to pH 3, and extracted several times with ether. Recovery of the solvent left a considerable quantity (1.5 gm.) of a dark oil ($n = 1.4882^{27.5}_{D}$), with a strong odour of the essential oil. After mild acid hydrolysis the residual liquor gave a heavy red precipitate with Fehling's solution and yielded a mixture of osazones that were not separately identified.

"INSOLUBLE" RESIDUE V (see Diagram I)

The residue contained 35.0% of lignin (16), 0.47% of nitrogen, and 40.1% of ash. Cellulose was identified microchemically.

Conclusion

The following approximate composition of the gum has been calculated from the above data.

	%
Fatty acid esters	50
α-Amyrin	26
Polyisoprene	6
Ash	4
Lignin	3
Cellulose	2
Protein (N × 6.25)	0.8
Free fatty acids	0.2
Sterol	0.1
Resene	0.1
Essential oil	0.1
Calcium tartrate	Trace
Unidentified	7.7
	100.0

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